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Dissecting the Paracrine Interactions Contributing to Normal Testicular Function and During the Ageing Process

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Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

Michael Kings Curley

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Abstract

The mammalian testis is divided into two distinct compartments which carry out its principal functions. Spermatogenesis occurs within the seminiferous tubules and androgen biosynthesis primarily occurs in the interstitial space. Both these processes are entirely dependent upon the two major testicular somatic cell populations - the Sertoli and Leydig cells respectively. In human males, testicular spermatogenic and endocrine function declines during the ageing process. Of particular significance is the reported age-related decrease in Leydig cell androgen production as androgens have been suggested to play a crucial role in supporting lifelong general health in men, with low circulating testosterone linked to an increased risk of developing chronic age-related cardiometabolic diseases. However, the relationship between ageing, testicular function and disease is not fully understood, impeding the development of novel therapeutic strategies to treat age-related testicular dysfunction.

In one set of studies undertaken herein, a series of novel mouse models of premature ageing were utilised to begin to dissect the process of age-related testicular degeneration. Firstly, a novel knockout-first conditional allele of a previously reported premature-ageing model driven by *Cisd2* (CDGSH Iron Sulphur Domain 2) deficiency was validated and the testicular phenotype characterised and compared to that of naturally aged mice at 18-months of age. Histological analyses revealed premature testicular atrophy at 6-months of age in CISD2 deficient mice, consistent with observations of the naturally aged testis. Circulating testosterone was significantly lower in CISD2-deficient mice compared to wild-type controls at 6-months of age and the luteinising hormone/testosterone ratio was significantly elevated, indicative of compensated Leydig cell failure. *mRNA* expression of key genes involved in androgen production were also significantly reduced in the CISD2-deficient testis, pointing to Leydig cell dysfunction in this model of premature aging. Next, Cre/*LoxP* technology was used to delete *Cisd2* from specific testicular cell populations to determine which cell types control/support Leydig cell function during the ageing process. Testosterone production was unaffected when *Cisd2* was disrupted in either the Leydig cell population or Sertoli cell population. These observations suggest that disruption to the testicular microenvironment in which Leydig cells reside, rather than intrinsic Leydig cell ageing, may play a significant role in age-associated Leydig cell dysfunction.

A second set of studies were carried out to investigate the role of leukemia inhibitory factor (LIF) signalling in the maintenance of testicular function. LIF is a pleiotropic cytokine belonging to the interleukin-6 family. In the rodent testis, LIF is expressed in fetal life and adulthood; the peritubular myoid cells thought to be the main site of production. Given their anatomical location within the testis, LIF produced by peritubular myoid cells may act on both intratubular and interstitial cells to influence spermatogenesis and steroidogenesis respectively. Indeed, LIFR is expressed in germ cells, Sertoli cells, Leydig cells as well as testicular macrophages suggesting that LIF may be a key paracrine regulator of testicular function. However, the precise role of LIF/LIFR signalling in the testis is largely unknown. As such, models of testicular cell-specific *Lifr* deletion were generated using Cre/*LoxP* technology. Analysis of these novel models of conditional LIFR ablation revealed that LIFR is dispensable in germ cells for normal spermatogenesis. However, LIFR ablation from Sertoli cells resulted in a progressive degenerative phenotype, characterised by abnormal germ cell loss, sperm stasis, seminiferous tubule distention and subsequent atrophy of the seminiferous tubules.

In a final set of studies, a rat model of Leydig cell ablation-regeneration was used to determine the regenerative capacity of human adipose-derived perivascular stem cells (hAd-PSC) as a potential therapy for testicular dysfunction. Following ethane dimethanesulphonate (EDS) mediated Leydig cell ablation, primary hAd-PSCs, cultured with or without LH, IGF-1, PDGFBB, T_3 and ITS supplement, were transplanted into the rat testis and Leydig cell regeneration was monitored *via* serial measurements of circulating luteinising hormone (LH) and testosterone. Overall, hAd-PSCs had no impact on the recovery of circulating testosterone levels. However, when pre-cultured with the cocktail of hormone/growth factor supplements, the LH spike induced by the removal of testosterone negative feedback was dampened, suggesting the transplanted cells may promote Leydig cell regeneration. Whether these cells differentiate into Leydig cells, or simply provide paracrine support to the regenerating Leydig cells remains to be determined. Although Ad-PSCs may enhance regeneration kinetics, the transplanted cells were undetectable in the testis 5 weeks post transplantation suggesting they may not survive in the context of long term xenogeneic transplantation.

Lay Summary

In males, the testicles have two main functions; to produce sperm *via* the process of spermatogenesis, and to produce the hormone testosterone. Both these functions rely on specific types of cells in the testis. The Sertoli cells provide important support to the developing sperm cells, and the Leydig cells are responsible for producing testosterone. As males age, the testicles become less efficient at producing sperm and testosterone. Reduced testosterone production in ageing men is particularly important because, further to its role in the reproductive system, testosterone is also required to keep other parts of the body healthy and low levels of testosterone have been linked to an increased risk of developing health problem such as diabetes and cardiovascular disease. However, the reasons why the testicles become less efficient during ageing and disease are not fully understood, making the development of new treatments to promote testicle function difficult.

To try and better understand what causes age-related testicular decline, the studies described in this thesis utilised a series of new mouse models of premature testicular ageing which were designed to limit 'ageing' to specific cells in the testicles. These models enabled the contribution of the different cell populations to the overall function of the testicles to be assessed. It was found that ageing of the Leydig cells themselves wasn't responsible for the decline in testosterone production but instead, changes to the local environment inside the testicle might be the reason they become less efficient as they age.

In a second set of experiments, signalling of a molecule known as leukemia inhibitory factor (LIF) was manipulated to see how it might regulate testicular function. This molecule is present in the testicle throughout life and is thought to be important, but its function has not been established. By disrupting LIF signalling in developing sperm cells and/or Sertoli cells, it was found that LIF signalling in the developing sperm cells is not needed for the production of mature sperm. In contrast, LIF signalling in Sertoli cells is needed to maintain normal spermatogenesis. These were the first experiments describing how LIF signalling may support testicle function.

A final set of experiments were carried out to try and find a new treatment to promote testicle function. Stem cells from human fat tissue were injected into the testicles of rats that had had their Leydig cells destroyed by a toxic chemical. The results showed that the stem cells might help restore testosterone production, but they do not survive long-term in the testicles which may limit their use as a therapy.

Presentations Relating to This Thesis

BBSRC/EastBio Symposium (Dundee, Scotland) – Poster Presentation, Aug, 2014.
Establishing the Cause-Consequence Relationship between Androgens and the Normal Ageing Process.
Michael Curley[1], Laura Milne[1], Alistair Elfick[2], Patrick Hadoke[3], Paul Potter[4] and Lee B. Smith[1].

Scottish Cardiovascular Forum (Edinburgh, Scotland) – Poster Presentation, Feb 2015.
Establishing the Cause-Consequence Relationship between Androgens and the Normal Ageing Process.
Michael Curley[1], Laura Milne[1], Alistair Elfick[2], Patrick Hadoke[3], Paul Potter[4] and Lee B. Smith[1].

European Testis Workshop (St. Malo, France) – Poster/Oral Presentation, June 2016.
Compensated Leydig Cell Failure in a Novel Mouse Model of Premature Ageing.
Michael Curley[1], Laura Milne[1], Alistair Elfick[2], Patrick Hadoke[3], Paul Potter[4] and Lee B. Smith[1].

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1 Literature Review

1.1 Testicular Development

1.1.1 The Male Genetic 'Switch'

Establishment of the phenotypic male begins with the commitment of the bipotential gonad to form a testis. This process relies on the presence of a Y chromosome, which harbours the crucial *Sry* (Sex-determining Region, Y chromosome) gene (Sinclair *et al.*, 1990). In the absence of SRY (i.e. in XX females, or XY individuals with mutated/deleted *Sry*), ovaries rather than testes develop (Jager *et al.*, 1990, Hawkins *et al.*, 1992, Kato *et al.*, 2013). Conversely, transgenic *Sry* expression in genetically female mice results in testis development (Koopman *et al.*, 1991). In the mouse, *Sry* is expressed in pre-Sertoli cells, derived from the coelomic epithelium (Karl and Capel, 1998), from embryonic day (e) 10.5 to 12.5, reaching a peak at e11.5 throughout the length of the gonad (Bullejos and Koopman, 2001, Sekido *et al.*, 2004, Wilhelm *et al.*, 2005). A crucial function of SRY appears to be up-regulation of *Sox9* (SRY box-9) (Sekido *et al.*, 2004, Wilhelm *et al.*, 2005, Hiramatsu *et al.*, 2009), a key transcription factor required for Sertoli cell differentiation and subsequent testicular development as evidenced by sex reversal in *Sox9* gain of function XX and loss of function XY gonads (Vidal *et al.*, 2001, Barrionuevo *et al.*, 2006, Lavery *et al.*, 2011). SOX9 upregulates the expression of *Fgf9* (Fibroblast Growth Factor-9) and together FGF9 and SOX9 act to inhibit the expression of pro-ovarian genes including *Wnt4* (Wingless-related MMTV integration site 4). Recent research has highlighted a large number of target genes either activated or repressed by SRY and SOX9 (Li *et al.*, 2014), but ultimately the development of the bipotential gonad into a testis or ovary depends on the balance of *Fgf9* and *Wnt4* expression (Kim *et al.*, 2006). A schematic overview of testicular development is presented in Figure 1.1.

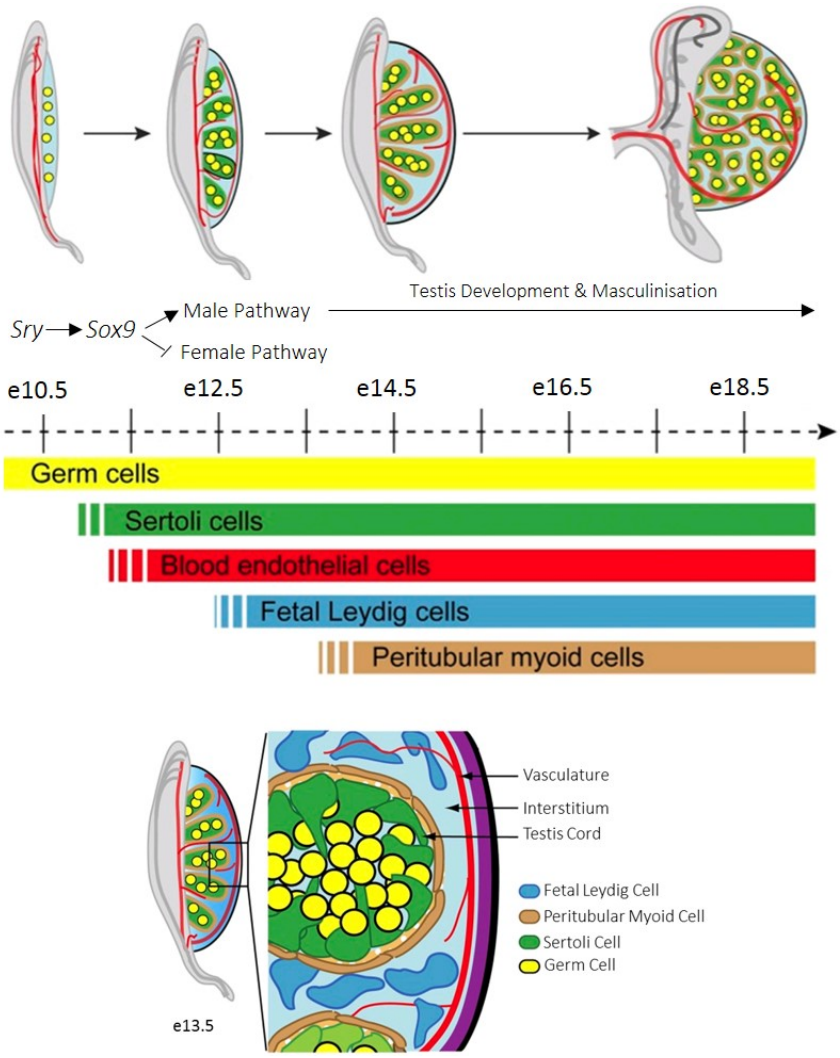


Figure 1.1 Overview of Testicular Development in the Mouse. *Sry* (Sex-determining Region, Y chromosome) is expressed in pre-Sertoli cells from e10.5 and functions to upregulate *Sox9* (SRY-Box 9), inducing Sertoli cell differentiation and subsequent testicular development. Testis cords begin to form at embryonic day (e) 11.5 representing the first morphological change associated with testicular development. From e12.5, fetal Leydig cells appear in the interstitium and are required for subsequent masculinisation and development of the male reproductive tract. This figure has been adapted from Svingen and Koopman (2013) in accordance with Creative Commons Licensing (Attribution-NonCommercial 3.0 Unported).

1.1.2 Formation of the Testis

Formation of the testis cords between e11.5 and 12.5 represents the first major morphological change associated with testis development (Nel-Themaat *et al.*, 2009) and is dependent on migration of endothelial cells from the mesonephros (Buehr *et al.*, 1993, Merchant-Larios *et al.*, 1993, Tilmann and Capel, 1999, Cool *et al.*, 2008,

Combes *et al.*, 2009). Testis cords comprise Sertoli cells and germ cells surrounded by a layer of peritubular myoid cells and are the precursor to the spermatogenic seminiferous tubules of the adult testis. Migrating endothelial cells contribute to the formation of the testicular vasculature, a process which is critical for formation of seminiferous cords (Brennan *et al.*, 2003, Yao *et al.*, 2006, Coveney *et al.*, 2008, Combes *et al.*, 2009, Cool *et al.*, 2011).

Concomitant with the establishment of the testicular vasculature and formation of the seminiferous cords, fetal Leydig cells appear in the interstitial compartment of the mouse testis at e12.5-13.0 (Greco and Payne, 1994, Baker *et al.*, 1999, Yao *et al.*, 2002). Fetal Leydig cells develop from a heterogeneous population of interstitial precursors derived from both the coelomic epithelium and cells of the gonad-mesonephros border (DeFalco *et al.*, 2011, Liu *et al.*, 2016) under the influence of various signalling molecules including Sertoli cell-derived desert hedgehog (DHH) and platelet derived growth factor (PDGF). In mice, disruption of *Dhh* (Yao *et al.*, 2002) and platelet derived growth factor receptor-alpha (*Pdgfra*) (Brennan *et al.*, 2003) results in defective fetal Leydig cell development. Conversely, constitutive activation of the hedgehog pathway in SF1-positive somatic cells of the mouse ovary results in the development of functional fetal Leydig cells as evidenced by the virilisation of XX embryos (Barsoum *et al.*, 2009), highlighting the importance of fetal Leydig cells in the process of masculinisation (see section 1.1.3). Notch-*Hes1* (*H*airy *E*nhancer of *S*plit-1) signalling has also been implicated in the regulation of fetal Leydig cell development. Pharmacological and genetic blockade of Notch signalling results in increased fetal Leydig cell numbers, while constitutive Notch activation impairs Leydig cell development and results in increased undifferentiated mesenchymal cells (Tang *et al.*, 2008). Additionally, abnormal testis cords are noted in both loss and gain of function models, suggesting interstitial cells of the fetal gonad may influence cord formation. Indeed, fetal Leydig cells produce activin-A, a member of the transforming growth factor beta (TGF-β) protein family, which acts on Sertoli cells to promote their proliferation and expansion of the testis cords (Archambeault and Yao, 2010). The expression of leukemia inhibitory factor (LIF) and its receptor (LIFR) coincides with the development of the fetal Leydig cell population. Interestingly, *Lif* is upregulated in the embryonic testis as a result of bis (2-ethylhexyl) phthalate (DEHP) exposure and

LIF promotes fetal Leydig cell aggregation (Lin *et al.*, 2008), a common feature of phthalate-induced testicular dysgenesis (Mahood *et al.*, 2005, Lara *et al.*, 2017, van den Driesche *et al.*, 2017). However, a precise role for LIF/LIFR signalling in testicular development has not been established.

1.1.3 Masculinisation

Following commitment of the bipotential gonad to testis development, concerted action of Leydig cell-derived insulin-like factor 3 (INSL3)(Kubota *et al.*, 2001, Nef and Parada, 1999), Sertoli cell-derived anti-Müllerian hormone (AMH) (Behringer *et al.*, 1994, Munsterberg and Lovell-Badge, 1991) as well as the adequate production and action of androgen (Lyon and Hawkes, 1970, Geissler et al., 1994, Caron et al., 1997, Ahmed *et al.*, 2000, Hu *et al.*, 2002, Yeh *et al.*, 2002, Notini *et al.*, 2005, Hannema *et al.*, 2006, Welsh *et al.*, 2008) is required for masculinisation and development of the male reproductive tract. Androgens produced by the fetal testis function to stabilise the Wolffian ducts and promote proper development of the male reproductive tract and external genitalia (e.g. testis descent/penile morphogenesis), as well as masculinisation of other aspects of the fetus including the brain (Sato *et al.*, 2004, Welsh *et al.*, 2008, Welsh *et al.*, 2009b). Studies in rats have identified a critical window, between e15.5 to e18.5, in which androgens are crucial for the masculinisation process (Welsh *et al.*, 2008). Such a window is thought to exist during human fetal development between gestational weeks 8-12. Insufficient androgen production/action during this developmental window can give rise to malformations of the male reproductive system such as cryptorchidism (incomplete descent of the testes into the scrotum) and hypospadias (malformations of the penis). These developmental disorders, as well as adult onset of testicular germ cell cancer and reduced sperm counts are thought to comprise a testicular dysgenesis syndrome which share a common fetal origin (Boisen *et al.*, 2001, Skakkebaek *et al.*, 2001, Hughes *et al.*, 2006). In fact, an unfavourable environment *in utero* has been associated with lower adult testosterone levels, suggesting aspects of adult Leydig cell function may be ‘programmed’ during fetal life (Cicognani *et al.*, 2002, Vanbillemont *et al.*, 2010). In this regard, a recent study by Kilcoyne *et al.* (2014) suggests that insufficient androgen action on adult Leydig cell stem/progenitor cells during embryonic development may negatively impact the function of the adult Leydig cell population.

1.2 Structure and Function of the Adult Testis

The mammalian testis is a complex multicellular organ, separated into two distinct compartments (Figure 1.2) that carry out its principal functions: spermatogenesis and steroidogenesis.

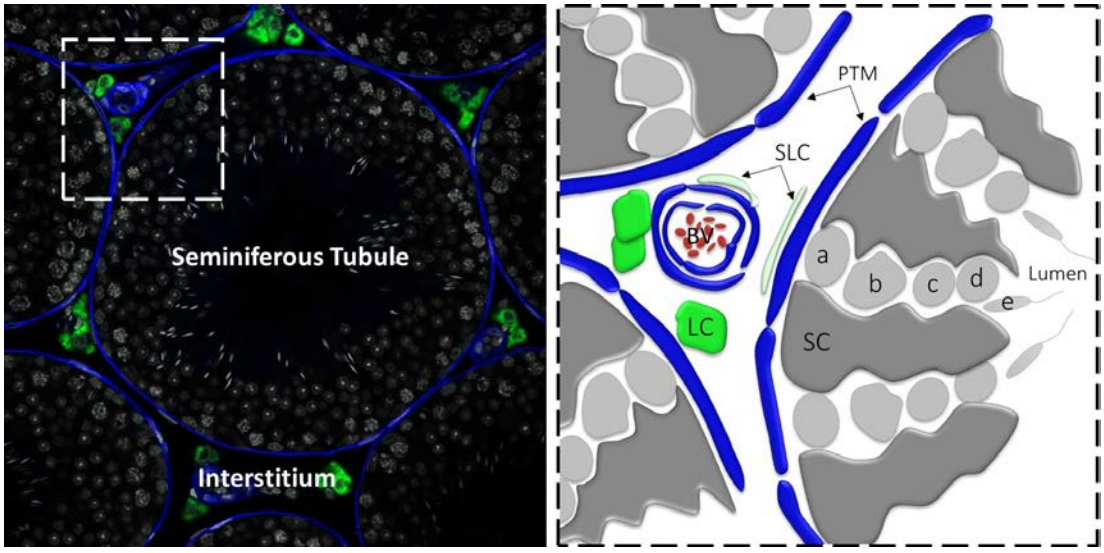


Figure 1.2 Structure of the Adult Testis. Cross Section of an adult mouse testis (left) and schematic representation (right) of the various cellular components of the adult testis. The testis can be divided into two distinct compartments; the tubular compartment is the site of spermatogenesis and consists of seminiferous tubules in which Sertoli (SC) and germ cells are present (a = spermatogonia; b = spermatocyte; c and d = round/elongating spermatids; e = elongate spermatids). The interstitial compartment, between the seminiferous tubules, is where the testosterone producing Leydig cells (LC) are found. PTM = peritubular myoid cell; SLC = stem Leydig cell; BV = blood vessel.

1.2.1 Leydig Cells

Leydig cells; the androgen producing cells found in the testis interstitium (Figure 1.2), were first described by Franz von Leydig in 1850 (Schneider, 2012). In rodents, two populations of Leydig cells (fetal and adult) arise during development (Lording and de Kretser, 1972, Tapanainen *et al.*, 1984, Zirkin and Ewing, 1987, Shima *et al.*, 2015). Regression of the fetal Leydig cell population, at least functionally, occurs between birth and postnatal day (d) 10-20 (O'Shaughnessy *et al.*, 2002b, Wu *et al.*, 2010). Whether fetal Leydig cell death occurs prior to replacement by the adult Leydig cell population is not entirely clear. For example, Yokoi *et al.* (1998) reported Leydig cell death in the perinatal rat testis based on immuno-detection of terminal

deoxynucleotidyl transferase whereas no apoptotic cells are detected in the testis interstitium during postnatal development in the mouse (Faria *et al.*, 2003). In line with the latter, fetal Leydig cells are reported to persist in the adult testis (Mendis-Handagama *et al.*, 1987, Kerr and Knell, 1988, Kaftanovskaya *et al.*, 2015, Shima *et al.*, 2015). In contrast to rodents, Leydig cell development in humans has been described as a 'tri-phasic' process evidenced by increases in testosterone and INSL3 production 2-3 months after birth (Forest *et al.*, 1973, Prince, 1990, Prince, 2001, Bay *et al.*, 2007). Leydig cell development has been extensively characterised using rodent models thus development of the adult Leydig cell population is described in the following section with reference primarily to the rat and mouse.

1.2.1.1 Adult Leydig Cell Development

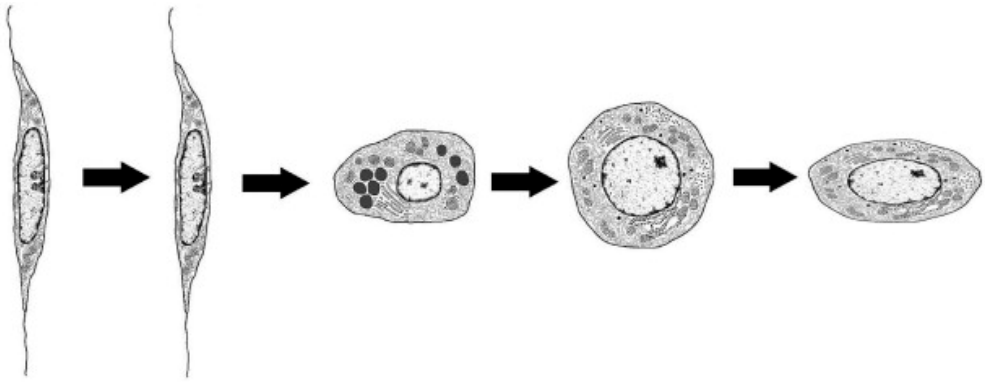
Adult Leydig cells begin to populate the testis during pubertal development however, the specific mechanisms regulating adult Leydig cell differentiation are not entirely understood. Early studies of ethane dimethanesulphonate (EDS) mediated adult Leydig cell ablation and regeneration in the rat proposed that, much like the development of fetal Leydig cells (see section 1.1.2), adult Leydig cells arise from an interstitial stem cell population (Kerr *et al.*, 1985, Jackson *et al.*, 1986). Indeed, numerous studies have since confirmed that adult Leydig cells arise from a spindle-shaped mesenchymal stem cell pool which is established in the testis interstitium during fetal life (Siril Ariyaratne *et al.*, 2000, Davidoff *et al.*, 2004, Ge *et al.*, 2006, O'Shaughnessy *et al.*, 2008, Stanley *et al.*, 2012, Jiang *et al.*, 2014, Kilcoyne *et al.*, 2014, Li *et al.*, 2016, Liu *et al.*, 2016, Zang *et al.*, 2017). However, it is not clear whether this stem cell population is common to both fetal and adult Leydig cells. For example, common signalling molecules including DHH and PDGF-A are involved in the development of both fetal and adult Leydig cell populations supporting the hypothesis of a common precursor to both populations (Clark *et al.*, 2000, Gnessi *et al.*, 2000, Yao *et al.*, 2002, Brennan *et al.*, 2003, Park *et al.*, 2007, Barsoum *et al.*, 2013). However, divergence in the development and/or function also exists between fetal and adult Leydig cells. For instance, whilst fetal Leydig cells develop independently of luteinising hormone (LH) signalling, proper establishment of the adult population does not occur in the absence of LH stimulation (Shan *et al.*, 1995, O'Shaughnessy *et al.*, 1998, Baker and O'Shaughnessy, 2001, Zhang *et al.*, 2001, Ma

et al., 2004). Additionally, fetal, but not adult, Leydig cells express the melanocortin type 2 receptor and are thus sensitive to adrenocorticotrophic hormone which stimulates fetal Leydig cell steroidogenesis (O'Shaughnessy *et al.*, 2003, Johnston *et al.*, 2007). Whether these discrepancies refute a common ontogeny for fetal and adult Leydig cells, or simply reflect temporal differences in the regulation of Leydig cell development/function remains to be established. Interestingly, there is evidence to suggest that two separate subsets of Leydig cells exist in the adult mouse testis which show differential phenotypic responses to perturbations in paracrine signalling (Payne *et al.*, 1980, Welsh *et al.*, 2012). Increasing our understanding of the regulatory mechanism(s) governing the development and function of the adult Leydig cell population will aid the development of novel regenerative therapies to replace poorly functioning aged Leydig cells.

1.2.1.1.1 Stem Leydig Cells

Development of the adult Leydig cell population progresses through four distinct stages (stem – progenitor –immature – adult; Figure 1.3) relating to alterations in cell morphology, gene expression and steroidogenic activity (O'Shaughnessy and Murphy, 1991, Ge and Hardy, 1998, Ge *et al.*, 2005, Dong *et al.*, 2007). The precise origin of stem Leydig cells is a topic of contentious debate, with evidence of peritubular (Siril Ariyaratne *et al.*, 2000) and/or perivascular (Davidoff *et al.*, 2004) origins reported in the literature. Ge *et al.* (2006) described a population of spindle-shaped cells in the d7 rat testis interstitium, primarily in association with the peritubular region, which were negative for the Leydig cell lineage markers 3β-hydroxysteroid dehydrogenase (HSD3b) and luteinising hormone/chorionic gonadotrophin receptor (LHCGR). These cells were isolated based on negative selection for LHCGR and positive selection for PDGFRa and were further shown to express the stem cell markers leukemia inhibitory factor receptor (LIFR) and c-Kit. The authors went on to demonstrate these cells are capable of long term self-renewal *in vitro* and can differentiate into testosterone-producing Leydig cells *in vitro* and *in vivo,* suggesting that this cell population may, in the rat testis, represent the elusive stem Leydig cells. More recently, using a green fluorescent protein-tagged nestin (*Nes-GFP*) mouse, Jiang *et al.* (2014) isolated a population of cells from the postnatal mouse testis with similar characteristics and also suggested that cluster of differentiation-51 (CD51) may be a putative surface marker

of stem Leydig cells in the postnatal mouse testis. Indeed, Zang *et al.* (2017) went on to show that CD51-positive cells isolated from 12-week old mouse testes display stem cell characteristics and are able to differentiate into functional Leydig-like cells both *in vitro* and *in vivo.* Using a linage tracing approach, Kilcoyne *et al.* (2014) suggested that the orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor-II (COUP-TFII) is a marker of adult Leydig stem/progenitor cells. COUP-TFII is essential for the development of adult Leydig cells during pubertal development. Using an inducible gene ablation approach, Qin *et al.* (2008) demonstrated that COUP-TFII deficiency resulted in arrested Leydig cell development at the progenitor stage.



CELLULAR ATTRIBUTES	STEM LEYDIG CELL	PROGENITOR LEYDIG CELL	IMMATURE LEYDIG CELL	ADULT LEYDIG CELL	AGED LEYDIG CELL
Mitotic activity	Low	high	Once	Probably none	Probably none
Morphology	Spindle	Spindle	Round with large lipid droplets	Round with small lipid droplets	Round with small lipid droplets
Smooth ER	Low	Low	High	Highest	High
Testosterone Metabolism	None	High	Highest	Low	Low
Testosterone Synthesis	None	Low	Intermediate	High	Intermediate
Age when cell is typical	7 days	21 days	35 days	90 days	20 months
LH Receptor Number	None	Low	Intermediate	High	Intermediate

Figure 1.3 Stages of Adult Leydig Cell Development. Adult Leydig cells develop through stem, progenitor and immature stages before becoming mature, fully functional adult Leydig cells. Each developmental stage is characterised by alterations in cell morphology, gene expression and steroidogenic activity. With age, adult Leydig cells become dysfunctional. N.B. the ages given in this schematic are based on development of Leydig cells in the rat testis. This image has been modified from Chen *et al.* (2009b).

1.2.1.1.2 Progenitor to Mature Leydig Cells

In the rat testis, stem Leydig cells begin to express LHCGR and commit to the adult Leydig cell lineage by d14, giving rise to progenitor Leydig cells which express LHCGR as well as the steroidogenic enzymes P450 cholesterol side-chain cleavage enzyme (CYP11A1), HSD3B and 17α-hydroxylase, 17,20-lyase (CYP17A1) (Shan and Hardy, 1992, Shan *et al.*, 1993, Ge *et al.*, 2006, Teerds *et al.*, 2007a). As such, progenitor Leydig cells have the capacity for steroidogenesis, with androsterone being the primary product (Ge and Hardy, 1998). The transition of progenitor Leydig cells into immature Leydig cells, at approximately d35 in both rats and mice (Wu *et al.*, 2010), is characterised by a transformation from spindle-shaped to a more rounded morphology, expansion of the smooth endoplasmic reticulum (SER) acquisition of numerous large cytoplasmic lipid droplets and increased steroid metabolising activities (Shan *et al.*, 1993, Haider *et al.*, 2007, Chen *et al.*, 2009b). Immature Leydig cells produce more testosterone than do progenitor Leydig cells however; they primarily produce androstanediol (Ge and Hardy, 1998). The final transition into mature adult Leydig cells is characterised by further expansion of the SER, a decrease in lipid droplets, altered expression of androgen metabolising enzymes and subsequent increase in testosterone production (Zirkin and Ewing, 1987, Shan *et al.*, 1993, Ge and Hardy, 1998, Baker *et al.*, 1999, Wu *et al.*, 2010).

1.2.1.2 Regulation of Leydig Cells

As mentioned in section 1.2.1.1, studies in mice lacking circulating LH or LHCGR highlight a requirement for LH signalling in the development and maturation of adult Leydig cells. For example; in the *Hpg* mouse, which does not produce gonadotrophin releasing hormone (GnRH) and subsequently LH, adult Leydig cell number is reduced by approximately 90% (Baker and O'Shaughnessy, 2001). The *Hpg* mouse is also devoid of follicle stimulating hormone (FSH) and treatment of *Hpg* mice with FSH results in an increase in Leydig cell number (O'Shaughnessy *et al.*, 2010a). Additionally, Leydig cell number is reduced in mice lacking FSH receptors (FSHR) suggesting a role for FSH in adult Leydig cell development (O'Shaughnessy *et al.*, 2012). However, in FSH-deficient mice no such difference is observed (Baker *et al.*, 2003). In this model, circulating LH levels are normal, whereas FSHR-deficient mice have supraphysiological levels, which may account for this discrepancy and suggests

that FSH *per se* is not required for Leydig cell development in the presence of normal LH. Interestingly, Baker *et al.* (2003) demonstrated constitutive activation of the FSHR under baseline conditions in MLTC-1 cells transfected with murine FSHR. Thus, basal FSHR activation in Sertoli cells may stimulate the production of trophic factors required for proper attainment of the adult Leydig cell population.

Indeed, Sertoli cells provide paracrine support required for the development and maintenance of adult Leydig cells (Rebourcet *et al.*, 2014a, Rebourcet *et al.*, 2014b). Whilst the full complement of Sertoli cell-derived factors mediating Sertoli-Leydig cell crosstalk remains to be established, DHH and PDGF have been implicated as important players. Adult Leydig cells fail to develop in both DHH and PDGF-A deficient mice (Clark *et al.*, 2000, Gnessi *et al.*, 2000) and both molecules have been shown to influence stem Leydig cell proliferation and differentiation *in vitro* (Odeh *et al.*, 2014, Li *et al.*, 2016). Furthermore, altered expression of *Dhh* and *Pdgfa* during EDS-mediated adult Leydig cell ablation and regeneration has been reported (O'Shaughnessy *et al.*, 2008). Additionally, platelet derived growth factor-beta (PDGF-B) has been shown to promote stem Leydig cell proliferation *in vitro*, while inhibiting their differentiation (Odeh *et al.*, 2014, Li *et al.*, 2016).

In addition to DHH and PDGF-A, O'Shaughnessy *et al.* (2008) also suggested that leukemia inhibitory factor (LIF) may play a role in adult Leydig cell development. LIF is a pleiotropic cytokine belonging to the IL-6 family and is produced by multiple cell types in the testis, particularly by the peritubular myoid cells (Piquet-Pellorce *et al.*, 2000). Leukemia inhibitory factor signals through a heterodimeric receptor complex composed of the leukemia inhibitory factor receptor (LIFR, also known as gp190), which binds LIF, and the signal transducing gp130 subunit common to the IL-6 family members (Gearing *et al.*, 1991, Gearing *et al.*, 1992, Ip *et al.*, 1992). As mentioned in section 1.2.1.1.1, stem Leydig cells express LIFR and LIF has been shown to stimulate proliferation of stem Leydig cells (Ge *et al.*, 2006). *In vitro* studies have implicated the closely related IL-6-type cytokine oncostatin M (OSM) in aspects of adult Leydig cell development and function (Teerds *et al.*, 2007b). Additionally, *in vitro* studies have demonstrated LIF can have both inhibitory and stimulatory effects on Leydig cell steroidogenesis (Mauduit *et al.*, 2001, Wang *et al.*, 2016b). However, the role of LIF

signalling in the testis remains to be determined. No testicular phenotype is reported in *Lif*-deficient mice (Stewart *et al.*, 1992), which may be attributed to redundancy between IL-6 family cytokines. In contrast, *Lifr* disruption results in perinatal death (Ware *et al.*, 1995). Thus, LIFR-deficient mice cannot be used for *in vivo* studies into the role of LIF/LIFR signalling in the development and function of the adult Leydig cell population.

Androgens are also thought to be necessary for adult Leydig cell development as Leydig cell number and function is significantly impaired both in androgen receptor knock-out (ARKO) and *Tfm* mice (Murphy *et al.*, 1994, O'Shaughnessy *et al.*, 2002a, De Gendt *et al.*, 2005, O'Shaughnessy *et al.*, 2012). However, the testes of ARKO and *Tfm* animals are cryptorchid, complicating the interpretation of androgen actions in adult Leydig cell development in these models. Furthermore, global androgen action is abolished in both ARKO and *Tfm* mice thus, assigning cell-specific effects of androgens on adult Leydig cell development is challenging. Conditional ablation of androgen receptors in Sertoli cells (De Gendt *et al.*, 2005), peritubular myoid cells (Welsh *et al.*, 2012) and Leydig cells (O'Hara *et al.*, 2015) suggest that paracrine and autocrine androgen action may impact aspects of Leydig cell development and maturation.

Development of the adult Leydig cell population may also be influenced by thyroid hormone. For example, experimentally-induced hypothyroidism *via* propyl-2-thiouracyl (PTU) during the neonatal/pre-pubertal period is reported to reduce the proportion of Leydig cells during early development (d12-16) whereas treatment with bioactive thyroid hormone (T_3) significantly increased Leydig cell numbers (Teerds *et al.*, 1998). More recently, studies in hypothyroid rats at older ages suggest that dietary-induced hypothyroidism delays, rather than blocks, adult Leydig cell development (Rijntjes *et al.*, 2009). The effect of thyroid hormone on Leydig cell regeneration following EDS-mediated ablation has been studied, although conflicting results are reported. Ariyaratne *et al.* (2000) reported that Leydig cell differentiation was inhibited in hypothyroid rats and accelerated in hyperthyroid rats. Conversely, Rijntjes *et al.* (2010) reported that the Leydig cell population returns to pre-treatment size in hypothyroid rats although regeneration kinetics may be slightly altered (i.e. a delayed wave of progenitor Leydig cell proliferation was observed in hypothyroid animals). It

is not clear whether the proposed effects of thyroid hormones are a result of direct signalling in developing Leydig cells or *via* paracrine signals from Sertoli cells which are also sensitive to thyroid hormones (Wagner *et al.*, 2008, Rijntjes *et al.*, 2017).

Insulin-like growth factor-1 (IGF-1) which belongs to the insulin family of growth factors has been shown to support the development and function of adult Leydig cells. Baker *et al.* (1996) reported an eighty percent reduction in circulating testosterone levels in *Igf1* null mice, accompanied by ultrastructural abnormalities indicative of retarded Leydig cell development. Altered proliferation and differentiation of adult Leydig cells, from the progenitor stage onwards, results in a reduction in adult Leydig cell number in IGF-1 deficient testes which is rescued upon administration of recombinant IGF-1 (Wang and Hardy, 2004, Hu *et al.*, 2010a). During the transition from immature to adult Leydig cell, a reduction in the androgen metabolising enzymes 3α-hydroxysteroid dehydrogenase (HSD3A) and 5α-reductase type-1 (SRD5A1) occurs, resulting in increased testosterone production characteristic of mature adult Leydig cells. In IGF-1 deficient mice, *Srd5a1* expression remains high suggesting that Leydig cell development is arrested at the immature stage (Wang *et al.*, 2003, Hu *et al.*, 2010a).

1.2.2 Sertoli Cells

Sertoli cells, the somatic component of the seminiferous epithelium (Figure 1.2), were first described by Enrico Sertoli in 1865 (Baratelli *et al.*, 2002). As discussed in section 1.1.1, emergence of the Sertoli cells is crucial for the development of a testis and subsequent establishment of a phenotypic male. In the postnatal testis, the primary role of the Sertoli cell is to create the optimal environment within the seminiferous tubule to maintain the germ cell niche and provide support to the germ cells as they develop into spermatids. Experimental/genetic manipulations, which alter the number of Sertoli cells in the adult testis, have demonstrated that the size of the Sertoli cell population determines germ cell number and daily sperm production (Orth *et al.*, 1988, Hess *et al.*, 1993, Simorangkir *et al.*, 1995, Meachem *et al.*, 1996, Auharek *et al.*, 2011). More recently, it has been suggested that the number of Sertoli cells also determines the number and/or function of adult Leydig cells (Rebourcet *et al.*, 2014a, Rebourcet *et al.*, 2014b, Rebourcet *et al.*, 2017) as well as influencing the

intratesticular vascular volume and subsequently the exchange of fluid between the interstitial and vascular compartments (Rebourcet *et al.*, 2016).

1.2.2.1 Sertoli Cell Development

In mice, immature Sertoli cells proliferate during late embryonic and early postnatal life, such that the full Sertoli cell population is established by d20 (Vergouwen *et al.*, 1991, Baker and O'Shaughnessy, 2001). Initial development of the Sertoli cell population occurs independently of gonadotrophins; however, establishment of the full Sertoli cell population requires the action of FSH as evidenced by a reduction in adult Sertoli cell number in *Hpg*, FSH- and FSHR-deficient mice (Baker and O'Shaughnessy, 2001, Johnston *et al.*, 2004). Furthermore, in rats treated with recombinant FSH (Meachem *et al.*, 1996) and in *Hpg* mice with transgenic expression of either FSH or a mutation activated FSHR (Allan *et al.*, 2004), Sertoli cell number is increased. Thyroid hormone signalling, through Sertoli cell thyroid hormone receptor alpha-1 (THRa1), has also been implicated in Sertoli cell development. Transient neonatal hypothyroidism or THRa1 knockout increases Sertoli cell proliferation resulting in increased Sertoli cell numbers (Simorangkir *et al.*, 1995, Holsberger *et al.*, 2005, Auharek *et al.*, 2011, Fumel *et al.*, 2012), whereas hyperthyroidism has been shown to reduce Sertoli cell proliferation and thus reduce final Sertoli cell numbers (van Haaster *et al.*, 1993, Auharek *et al.*, 2011). Interestingly, Pitetti *et al.* (2013) noted a reduction in Sertoli cells due to decreased proliferation when insulin/IGF signalling is disrupted in Sertoli cells. Phenotypic similarities between this model and the models of FSH/FSHR deficiency and hyperthyroidism described above prompted the authors to investigate whether there is an interaction between these signalling pathways. The response of Sertoli cell insulin receptor/IGF receptor deficient mice to transient neonatal hypothyroidism (i.e. increased testis weight) was similar to control mice whilst the response to neonatal hemi castration or recombinant FSH administration was blocked. This suggests that the effects of FSH, but not thyroid hormone, on immature Sertoli cells is supported by active Sertoli cell insulin/IGF signalling. Adequate action of androgen is also required for the attainment of normal Sertoli cell number. In *Tfm* and androgen receptor knockout mice, final Sertoli cell number is reduced (Johnston *et al.*, 2004, Tan *et al.*, 2005). This effect is thought to be due to alterations in androgen-dependent paracrine factors as Sertoli cell

number is unaltered in Sertoli cell-specific androgen receptor knockout mice (De Gendt *et al.*, 2005). Which cell types mediate androgen actions on Sertoli cell proliferation is not entirely clear. Disruption of androgen receptors in peritubular myoid cells or Leydig cells does not result in altered Sertoli cell numbers however, androgen receptor ablation is not completely lost from these respective cell populations (Welsh *et al.*, 2009a, O'Hara *et al.*, 2015).

1.2.2.2 The Blood-Testis-Barrier

Following the phase of pubertal proliferation, Sertoli cells transform from their immature state into their mature form, taking on a number of specialised functions necessary for spermatogenesis to take place (Sharpe *et al.*, 2003, O'Shaughnessy *et al.*, 2007, Franca *et al.*, 2016). Mature Sertoli cells secrete fluid under the control of FSH which is required to nourish developing germ cells and aid their transport out of the testis (Jegou *et al.*, 1982, Richburg *et al.*, 1994, Rato *et al.*, 2010). Sertoli cell maturation is also associated with the development of the blood-testis barrier (BTB). Tight junctions, which consist of a number of junctional proteins including occludin and claudins (Saitou *et al.*, 2000, Mazaud-Guittot *et al.*, 2010), are formed between neighbouring Sertoli cells, which separates the basal, and adluminal regions of the seminiferous tubule (Dym and Fawcett, 1970). Together with immunosuppressive factors produced by Sertoli cells, the BTB restricts access to immune cells and other molecules to the adluminal compartment, thus creating an immunologically privileged site conducive to the development of auto-antigen expressing germ cells (Cesaris *et al.*, 1992, Mital *et al.*, 2010, Haverfield *et al.*, 2014, Terayama *et al.*, 2016). A number of factors regulate BTB function. Both FSH and androgen have been reported to modulate BTB permeability (Janecki *et al.*, 1991, Meng *et al.*, 2005). Additionally, cytokines also exert profound effects on BTB dynamics. For example, toxicant-induced disruption of the BTB is associated with a surge in TGFB3 (Lui *et al.*, 2003) and local administration of TGFB3 into the rat testis causes a transient increase in BTB permeability (Xia *et al.*, 2009). Similarly, injection of recombinant IL1 into the rat testis interstitium has been shown to impact BTB permeability (Sarkar *et al.*, 2008). Additionally, intratesticular injection of IL6 results in altered expression of occludin and claudin-11 and subsequent disruption to the seminiferous epithelium (Perez *et al.*, 2012). Interestingly, the IL6 signal transducing subunit glycoprotein 130 (gp130), as

well as other pleiotropic IL6-family members including LIF are expressed in the testis (Piquet-Pellorce *et al.*, 2000, Molyneaux *et al.*, 2003, Dorval-Coiffec *et al.*, 2005). Whilst IL6 and LIF signalling has been shown to be active in Sertoli cells *in vitro*, (Jenab and Morris, 1996, 1998, 2000), their precise role *in vivo* remains to be established.

1.2.3 Peritubular Myoid Cells

Peritubular myoid cells surround the seminiferous tubules (Figure 1.2) and, together with Sertoli cells, produce extracellular matrix (ECM) components including collagens, fibronectin and laminin which contribute to the basement membrane of the tubular wall (Skinner *et al.*, 1985, Richardson *et al.*, 1995, Flenkenthaler *et al.*, 2014). Peritubular myoid cells express the smooth muscle marker alpha actin (SMA) and are contractile to facilitate the movement of sperm toward the rete testis (Tung and Fritz, 1990, Palombi *et al.*, 1992, Tripiciano *et al.*, 1996, Tripiciano *et al.*, 1998, Rossi *et al.*, 2002). Additionally, the peritubular cells may also play a role in blood-testis barrier function. Early studies reported that junctions between peritubular cells could exclude a lanthanum tracer from the majority of the seminiferous tubules (Dym and Fawcett, 1970). It has since been shown that this function is likely supported by the Sertoli cell population; although peritubular myoid cells alone are able to prevent immune cells infiltrating the seminiferous tubules (Rebourcet *et al.*, 2014b).

Given their anatomical location, the peritubular myoid cells are well positioned to influence both interstitial and intratubular compartments. With regards to the former, disruption of androgen receptors in peritubular myoid cells results in compensated Leydig cell failure (increased LH to maintain testosterone production) suggesting androgen-dependent factors produced by peritubular cells promote Leydig cell function (Welsh *et al.*, 2009a). Peritubular myoid cells have also been suggested to play a role in the maintenance of the spermatogonial stem cell niche through androgen-stimulated production of glial-derived neurotrophic factor (GDNF) which mediates stem cell renewal (Chen *et al.*, 2014, Chen *et al.*, 2016). Leukemia inhibitory factor, first noted for its ability to inhibit the differentiation of M1 leukemic myeloid cell line (Ichikawa, 1969), is required to maintain the pluripotent state of embryonic stem cells *in vitro* (Smith *et al.*, 1988). LIF has also been shown to promote the proliferation and

survival of gonocytes in culture (De Miguel *et al.*, 1996). Using a bioassay based on the proliferative response of DA-1 cells to LIF, Piquet-Pellorce *et al.* (2000) demonstrated that peritubular myoid cells are the primary source of LIF within the rat testis. Additionally, Dorval-Coiffec *et al.* (2005) assayed the expression of *Lifr* in purified testicular cell populations and found that Leydig cells, Sertoli cells, peritubular myoid cells, spermatogonia and macrophages all express *Lifr*, whilst no expression was detected in spermatocytes and spermatids. The authors further characterised these cell populations using a flow cytometry approach to detect binding of biotinylated LIF. This revealed that, whilst all *Lifr*-expressing populations were able to bind LIF, spermatogonia displayed the highest level of LIFR expression. Finally, using an *in vitro* seminiferous tubule culture system, the authors also reported that LIF increased the number of [3H]-thymidine labelled cells after 72-hours of culture suggesting that LIF may stimulate spermatogonial proliferation and/or survival.

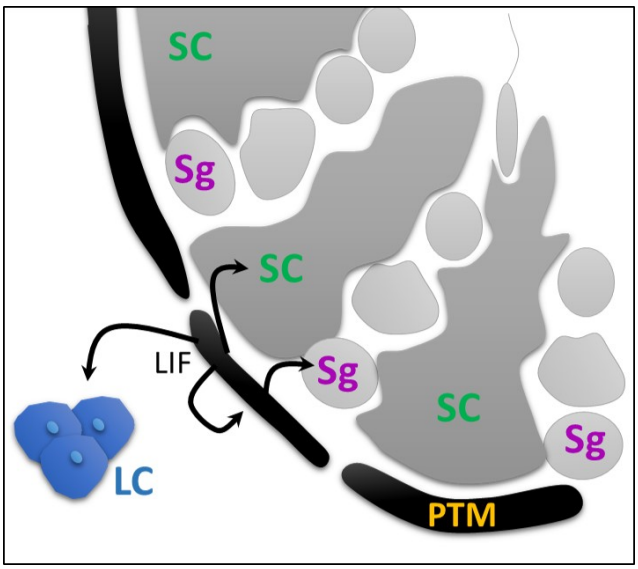


Figure 1.4 Leukemia Inhibitory Factor in the Testis. Peritubular myoid (PTM) cells are the predominant source of leukemia inhibitory factor (LIF) in the testis. The leukemia inhibitory factor receptor (LIFR) is widely expressed in the testis including in somatic Sertoli cells (SC) and Leydig cells (LC). Spermatogonia are considered a major target of LIF/LIFR signalling.

As mentioned in section 1.2.1.2, LIF may also play a role in Leydig cell development. Peritubular myoid cell-derived LIF may therefore be an important regulator of the peritubular stem Leydig cell niche. Further to its potential role in the development of the adult Leydig cell population, LIF may also regulate Leydig cell steroidogenic function. Mauduit *et al.* (2001) reported an inhibition of human chorionic

gonadotrophin (hCG)-stimulated testosterone production when LIF was added to primary porcine Leydig cell cultures. The authors further demonstrated that this was due to impaired cholesterol transport into the inner mitochondrial membrane, as LIF dose-dependently reduced hCG-stimulated steroidogenic acute regulatory (*Star*) expression. Furthermore, the inhibitory effect of LIF on maximal testosterone production was absent in cultures treated with 22R-hydroxycholesterol which can readily diffuse the mitochondrial membrane and serve as a substrate for steroidogenesis. Interestingly, in the above studies, LIF had no effect on basal testosterone production which is consistent with studies of mature rat Leydig cells (Wang *et al.*, 2016b). In contrast, Wang *et al.* (2016b) reported that LIF can stimulate basal testosterone production in immature rat Leydig cells through up-regulation of *Star* and *Hsd17b3* and down regulation *Akr1c14* and *Srd5a1* which are involved in testosterone metabolism. Together, these studies suggest that peritubular myoid cell-derived LIF may be an important paracrine regulator of testicular function. However, a role for LIF/LIFR signalling in the testis *in vivo* is yet to be described.

1.2.4 Germ Cells and Spermatogenesis

1.2.4.1 Spermatogonia & Mitosis

In the mature mammalian testis, haploid sperm are continually produced from diploid spermatogonial stem cells within the seminiferous tubules (Figure 1.2 and Figure 1.5) *via* the process of spermatogenesis. The sequence of spermatogenesis can be divided into mitotic, meiotic and post-meiotic phases. Spermatogonia, which comprise both stem and differentiating cell types, are located in the basal compartment between the basement membrane of the seminiferous tubule and the tight junctions of the blood-testis-barrier. During the mitotic phase, spermatogonia proliferate under the influence of various molecules including glial derived neurotrophic factor (GDNF) signalling *via* GDNF family receptor-alpha (GFRA)(Meng *et al.*, 2000); kit ligand (KITL) signalling *via* c-KIT receptor (Blume-Jensen *et al.*, 2000, Yoshinaga *et al.*, 1991) and Wnt/β-catenin signalling (Kumar *et al.*, 2016, Takase and Nusse, 2016, Chassot *et al.*, 2017). As mentioned in section 1.2.3, spermatogonial proliferation may also be influenced by LIF/LIFR signalling but *in vivo* evidence is lacking.

Incomplete cytokinesis during mitosis results in the formation of syncytial daughter spermatogonia (Dym and Fawcett, 1971, Moens and Hugenholtz, 1975), the significance of which is not entirely understood, but may promote synchronisation of subsequent cell divisions. Spermatogonia can thus be sub-divided into A_{single} (A_s), A_{paired} (A_{pr}) or $A_{aligned}$ (A_{al}) which are often referred to as undifferentiated spermatogonia (Figure 1.5). It was initially thought that stem cell activity is restricted to the A_s spermatogonia population (Huckins, 1971, Oakberg, 1971). However, it has since been proposed that whilst only a subset of A_s cells are spermatogonial stem cells, spermatogonia within cysts can recover stem cell potential *via* fragmentation (Nakagawa *et al.*, 2010, Hara *et al.*, 2014). Differentiating spermatogonia proceed through further mitotic divisions to produce A_1, A_2, A_3 and A_4, intermediate (In) and type B spermatogonia (Figure 1.5). Transition of B spermatogonia into primary spermatocytes is the last step of the mitotic phase of the spermatogenic cycle (Leblond and Clermont, 1952a).

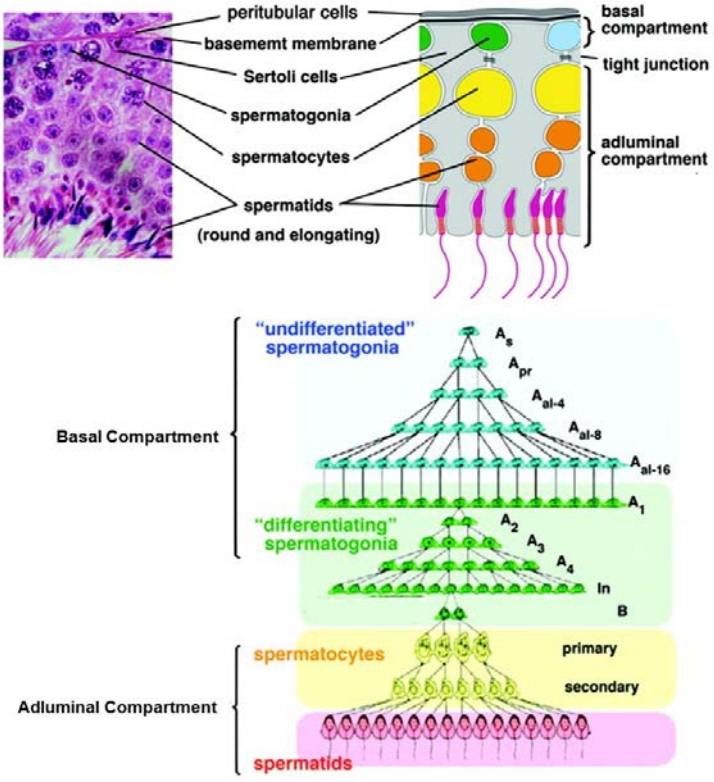


Figure 1.5 Overview of Spermatogenesis. On the left is an H&E stained mouse testis section showing the arrangement of germ cells within the seminiferous tubules. The schematic representation shown on the right highlights the location of the blood-testis-barrier. The mitotic phase of spermatogenesis takes place in the basal compartment whereas the meiotic and post-meiotic phases occur in the adluminal compartment. Modified from (Yoshida, 2010).

1.2.4.2 Spermatocytes & Meiosis

As discussed in section 1.2.2, junctional complexes between adjacent Sertoli cells within the seminiferous epithelium form the blood-testis barrier, which separates the basal and adluminal compartments (Figure 1.5). Primary spermatocytes must migrate across the BTB to the adluminal compartment where meiotic and post meiotic stages of spermatogenesis occurs. This is facilitated by a complex series of junctional degradation and restructuring (Mruk and Cheng, 2015).

A round of DNA replication occurs in primary spermatocytes prior to the initiation of meiosis, which proceeds in two rounds (meiosis I and meiosis II). Meiosis occurs in four phases (prophase, metaphase, anaphase and telophase). During prophase, DNA condenses, homologous chromosomes pair and recombination occurs, inducing genetic variation between developing germ cells. During metaphase and anaphase, chromosomes are separated and, in telophase they move to opposite poles of the cell prior to cell division. These processes are repeated during meiosis II, giving rise to 4 haploid round spermatids from each spermatocyte. Retinoic acid (RA), the active metabolite of vitamin A, stimulates the expression of *Stra8* (stimulated by retinoic acid) in pre-meiotic germ cells which is required for meiotic initiation as evidenced by the lack of meiotic spermatocytes in *Stra8*-deficient testes (Anderson *et al.*, 2008). The action of retinoic acid is balanced by its degradation *via* the activity of Cytochrome P450 Family 26 Subfamily B1 (CYP26B1) which functions to prevent meiosis in the fetal testis until the initiation of spermatogenesis (Bowles *et al.*, 2006, MacLean *et al.*, 2007).

1.2.4.3 Spermatids & Spermiogenesis

Following the meiotic phase of spermatogenesis, round spermatids undergo dramatic morphological changes, developing a distinctive head, mid-piece and tail as they transform into elongate spermatids during the process of spermiogenesis. The transition from round spermatids into elongate spermatids involves acrosome development, chromatin remodelling, a significant reduction in cytoplasmic volume and development of the characteristic sperm tail (Leblond and Clermont, 1952b, Oakberg, 1956). Acrosome development begins after the completion of meiosis II when pro-acrosomic granules accumulate and fuse (Ventela *et al.*, 2000, Kang-Decker

et al., 2001). As elongating spermatids develop, extensive chromatin remodelling takes place as histones are replaced by protamines which results in compaction of DNA (Kistler *et al.*, 1996, Braun, 2001, Cho *et al.*, 2001). Shaping of the sperm head is facilitated by a transient structure termed the manchette - a 'skirt-like' arrangement of microtubules originating just below the acrosome and projecting into the spermatid cytoplasm (Russell *et al.*, 1991, O'Donnell and O'Bryan, 2014). A second microtubule based structure called the axoneme develops during the post-meiotic phase of spermatogenesis and forms the core structure of the spermatid tail required for sperm motility. A mitochondria-rich sheath termed the mid-piece surrounds the axoneme and provides energy (Inaba, 2003). Cytoplasm is gradually displaced from the developing spermatid tail, forming a cytoplasmic lobule next to the spermatid head which is eventually expelled as a residual body and subsequently phagocytosed by Sertoli cells (Beardsley and O'Donnell, 2003, Russell, 1979). The final stage of spermiogenesis is the release of elongate spermatids into the seminiferous tubule lumen during the process of spermiation (O'Donnell *et al.*, 2011).

1.3 The Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.6) is a master endocrine regulator of testicular function. Gonadotrophin releasing hormone (GnRH) is produced by the hypothalamus and is released under stimulation by kisspeptins signalling through the kisspeptin receptor (GPR54) (d'Anglemont de Tassigny *et al.*, 2007, d'Anglemont de Tassigny *et al.*, 2008, Kirilov *et al.*, 2013). GnRH in turn stimulates gonadotrophs in the anterior pituitary to produce the gonadotrophins luteinising hormone (LH) and follicle-stimulating hormone (FSH). The *Hpg* mouse illustrates the importance of GnRH in the control of reproduction. In these mice, a mutation in the *Gnrh* gene results in the absence of GnRH, and subsequently gonadotrophins, leading to defective testicular development and function (Cattanach *et al.*, 1977, Mason *et al.*, 1986).

As discussed in section 1.4.1, LH binds to its receptor on Leydig cells to stimulate testosterone production. Circulating testosterone, either directly or *via* peripheral aromatisation to estradiol, feeds back to the hypothalamic-pituitary unit to decrease LH production (Matsumoto and Bremner, 1984, Sheckter *et al.*, 1989, Matsumoto,

1990, Hayes *et al.*, 2000, Pitteloud *et al.*, 2008). Follicle stimulating hormone binds to the follicle stimulating hormone receptor on Sertoli cells to promote spermatogenesis and testis function (discussed in sections 1.2.1.2 and 1.2.2). Inhibins, first isolated from ovarian follicular fluid (Ling *et al.*, 1985), are produced by Sertoli cells in response to FSH stimulation (Le Gac and de Kretser, 1982, Morris *et al.*, 1988, Pineau *et al.*, 1990, Sharpe *et al.*, 1988). Inhibins feedback to the pituitary to inhibit FSH production (Tilbrook *et al.*, 1993, Kishi *et al.*, 2000, Hayes *et al.*, 2001).

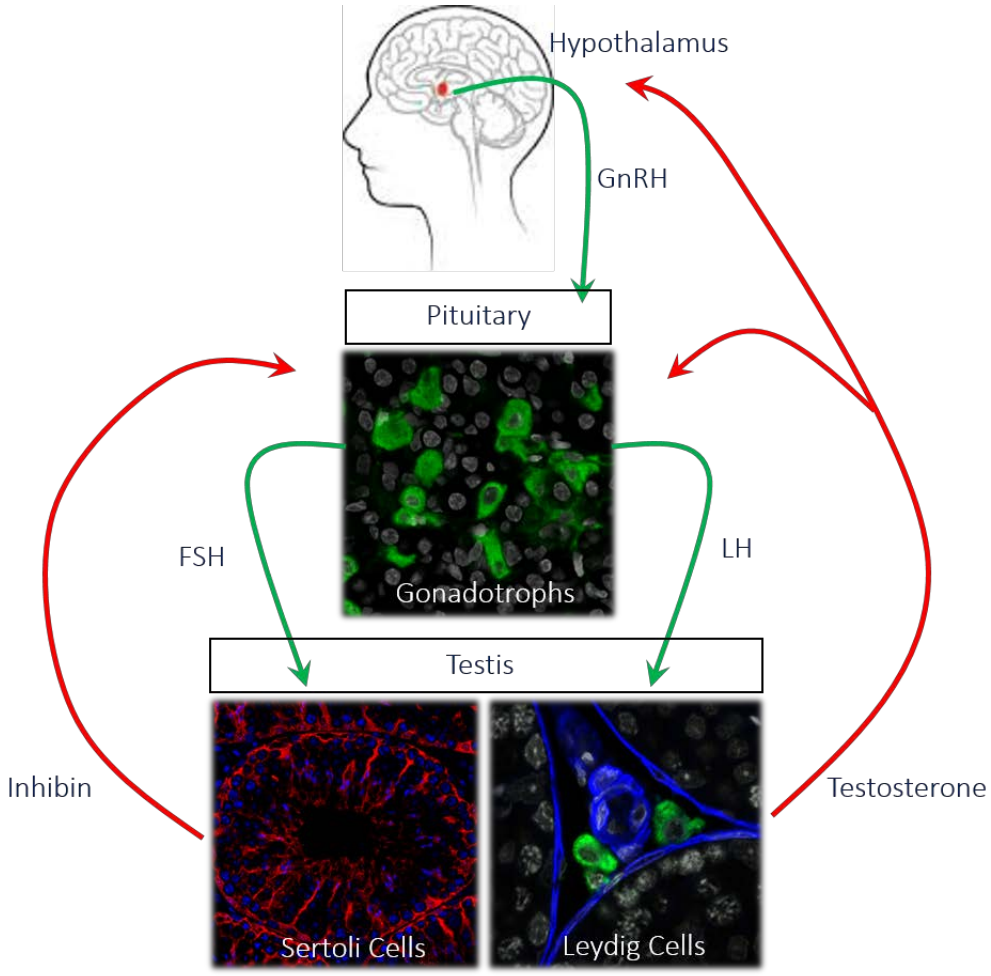


Figure 1.6 The Hypothalamic-Pituitary-Gonadal (HPG) Axis. Testicular function is under endocrine control by the HPG axis. Gonadotrophin releasing hormone (GnRH) is released from the hypothalamus and stimulates the production of luteinising hormone (LH) and follicle stimulating hormone (FSH) by pituitary gonadotrophs. LH and FSH bind to their receptors on Leydig (LH) and Sertoli (FSH) cells to stimulate steroidogenesis and spermatogenesis. Gonadotrophin-stimulated production of testosterone and inhibin feeds back to the hypothalamic-pituitary unit to negatively regulate gonadotrophin secretion. Green and red lines represent a stimulatory or inhibitory role respectively.

1.4 Androgen Production

Androgens (e.g. testosterone) are produced within testicular Leydig cells *via* the process of steroidogenesis. During steroidogenesis, a cascade of enzymatic reactions convert cholesterol, the common steroid hormone precursor, into sex steroid hormones.

1.4.1 Luteinising Hormone Receptor Signalling

Leydig cell androgen biosynthesis is regulated by pituitary-derived luteinising hormone (see section 1.3). Disruption of either the luteinising hormone-beta subunit (*Lhb*) or the luteinising hormone receptor (*Lhcgr*) results in dramatically reduced testosterone levels and defective testicular development (Lei *et al.*, 2001, Zhang *et al.*, 2001, Ma *et al.*, 2004, Zhang *et al.*, 2004). Luteinising hormone binds to its cognate G-protein coupled receptor (LHCGR) on the cell surface (McFarland *et al.*, 1989, Xie *et al.*, 1990). Binding of luteinising hormone to LHCGR stimulates the cyclic adenosine 3′,5′-monophosphate (cAMP) pathway which induces a rise in intracellular cAMP levels *via* the action of adenylyl cyclase. Increased cAMP in turn activates downstream effector molecules including protein kinase A (PKA) and protein kinase C (PKC) (Dufau *et al.*, 1977, McFarland *et al.*, 1989, Hansson *et al.*, 2000, Stocco *et al.*, 2005, Manna *et al.*, 2007). The cAMP pathway stimulates steroidogenesis through the activation of transcription factors including CREB (cAMP response elements binding protein) and GATA4 (GATA binding protein 4) which activate downstream target genes including *Scarb1, Star, Cyp11a1, Hsd3b1, Hsd3b6* and *Cyp17a1* (Manna *et al.*, 2002, Tremblay and Viger, 2003, Bergeron *et al.*, 2015). Phosphodiesterase (PDE) enzymes balance the activity of adenylyl cyclase by catalysing the breakdown of cAMP to regulate intracellular levels. Pharmacological and genetic manipulation of PDEs has been shown to increase the sensitivity of Leydig cells to LH/hCG-stimulated testosterone production (Dufau *et al.*, 1974, Shimizu-Albergine *et al.*, 2012).

1.4.2 Cholesterol Trafficking

A readily available pool of cholesterol is a prerequisite for steroid biosynthesis. To meet the demand of steroidogenic cells cholesterol can be synthesised *de novo*, mobilised from intracellular lipid droplets or obtained from circulating lipoproteins either by bulk-uptake or selectively *via* scavenger receptor B1 (SRB1) (Andersen and

Dietschy, 1978, Hu *et al.*, 2010b). The cascade of reactions that convert cholesterol into steroid hormones take place in the mitochondria and smooth endoplasmic reticulum. For steroidogenesis to occur, cholesterol must first be transferred across the mitochondrial membrane. This is dependent on the action of steroidogenic acute regulatory protein (StAR), deficiency of which impairs steroidogenesis (Clark *et al.*, 1994, Lin *et al.*, 1995, Caron *et al.*, 1997). The translocator protein (TSPO); previously called peripheral benzodiazepine receptor (PBR), may act in concert with StAR to facilitate cholesterol trafficking although experimental findings are conflicting. Initial *in vitro* studies suggested TSPO played a role in the movement of cholesterol between the outer to inner mitochondrial membranes (Krueger and Papadopoulos, 1990) and that its genetic disruption inhibited steroidogenesis due to defective cholesterol transfer (Papadopoulos *et al.*, 1997). However, it has recently been demonstrated that steroidogenesis is unaffected upon global or cell-specific *Tspo* ablation (Morohaku *et al.*, 2014, Tu *et al.*, 2014).

1.4.3 The Steroidogenic Cascade

Cholesterol is converted to pregnenolone within the mitochondria by the action of P450 cholesterol side-chain cleavage enzyme (CYP11A1) (Burstein and Gut, 1976, Simpson, 1979, Pelletier *et al.*, 2001). This step is common to, and essential for, the biosynthesis of all steroids. Severe steroid deficiency and 44,XY sex-reversal is observed in humans with *CYP11A1* mutations (Hiort *et al.*, 2005, al Kandari *et al.*, 2006) and, in *Cyp11a1*-deficient mice, lipids accumulate in steroidogenic tissues (Hu *et al.*, 2002).

Conversion of pregnenolone to testosterone occurs in the smooth endoplasmic reticulum through four reactions catalysed by hydroxysteroid dehydrogenase 3-beta (HSD3B), 17α-hydroxylase, 17,20-lyase (CYP17A1) and hydroxysteroid dehydrogenase 17-beta (HSD17B). Interestingly, an ultrastructural localisation study of steroidogenic enzymes in rat adrenal and testis suggested that, in contrast to steroidogenic cells of the adrenal cortex, conversion of pregnenolone to progesterone by HSD3B in Leydig cells may take place in the mitochondria rather than the smooth endoplasmic reticulum (Pelletier *et al.*, 2001). In the mouse, there are six HSD3B isoforms, with HSD3B1 and HSD3B6 predominant in the testis (Baker *et al.*, 1999).

In humans, deficiency of HSD3B results in impaired steroidogenesis and in males can disturb sexual development due to the absence of androgen (Burckhardt *et al.*, 2015).

Steroidogenic enzymes can act on and compete for multiple substrates. As such, pregnenolone can be converted into androgen by either the Δ4 or the Δ5 pathway which diverge at the point of pregnenolone metabolism (Conley and Bird, 1997). In the Δ4 pathway, pregnenolone is initially metabolised to progesterone by the action of HSD3B. Progesterone is then converted to 17α-hydroxyprogesterone and then androstenedione through the action of CYP17A1 (Nakajin and Hall, 1981, Zuber *et al.*, 1986). Conversely, in the Δ5 pathway, CYP17A1 acts first to convert pregnenolone to 17α-hydroxypregnenolone and then dehydroepiandrosterone (DHEA), which is then converted to androstenedione by HSD3B. It has been suggested that preferential utility of the Δ4 or the Δ5 pathway varies between species with the Δ4 being predominant in rodents and the Δ5 in humans (Brock and Waterman, 1999, Fluck *et al.*, 2003). However, the studies of Brock and Waterman (1999) used partially purified recombinant human and rat CYP17A1 and those of Fluck *et al.* (2003) utilised microsomal isolates of the human fetal testis. As such, spatiotemporal context, which may influence which pathway predominates, is lacking. For example, Sheffield and O'Shaughnessy (1988) reported variation in the pathways of pregnenolone metabolism across development in the mouse testis. The importance of CYP17A1 in androgen biosynthesis is highlighted by defective sexual development/46,XY sex-reversal in individuals with *CYP17A1* mutations (Geller *et al.*, 1997, Di Cerbo *et al.*, 2002, Camats *et al.*, 2015).

The final step in testosterone biosynthesis is the conversion of androstenedione to testosterone by HSD17B3. In humans, the loss of HSD17B3 results in defective sexual development/46,XY sex-reversal (Geissler *et al.*, 1994, Werner *et al.*, 2012). Interestingly, in rat testes devoid of Leydig cells, some HSD17B enzyme activity is retained which suggests expression may not be restricted to the Leydig cell population (O'Shaughnessy and Murphy, 1991). In contrast to steroidogenesis in adult mouse testis, testosterone production in the embryonic testis is a combined effort from the fetal Leydig and Sertoli cells (SC). Fetal Leydig cells do not express HSD17B3 that is required for the final conversion of androstenedione to testosterone. Instead,

androstenedione is the principal androgen produced by fetal Leydig cells and is converted to testosterone by the fetal Sertoli cells (O'Shaughnessy *et al.*, 2000, Shima *et al.*, 2013). Testosterone can be further metabolised to the more potent androgen dihydrotestosterone (DHT) or to estradiol *via* the action of 5α-reductase (SRD5A) or aromatase (CYP19A1) respectively.

1.4.4 Androgens Support Male General Health

Further to their established role in masculinisation and development of the male reproductive tract *in utero* (Welsh *et al.*, 2008, MacLeod *et al.*, 2010), and the maintenance of reproductive function/fertility in adulthood (Dohle *et al.*, 2003), androgens have also been implicated in aspects of bone, muscle and lipid metabolism. For example, a role for androgens in bone turnover has been suggested whereby androgens function to negatively regulate bone resorption (Kawano *et al.*, 2003). Furthermore, androgen deprivation therapy (i.e. chemical or surgical castration for the treatment of prostate cancer) is associated with a decrease in bone mineral density, decreased muscle strength and increased fat mass (Daniell *et al.*, 2000, Basaria *et al.*, 2002). Additionally, in studies of chemical castration and testosterone replacement in healthy young men, testosterone levels are positively correlated with muscle strength and volume and negatively correlated with fat mass and high-density lipoprotein (HDL) cholesterol (Bhasin *et al.*, 2001, Finkelstein *et al.*, 2013). As such, the overall general health, as well as reproductive health, of adult males is thought to be supported by testosterone and its metabolites.

Indeed, both cross-sectional and prospective large cohort studies of adult males report an inverse relationship between testosterone levels and the risk of age-related cardio-metabolic diseases (Muller *et al.*, 2005, Kupelian *et al.*, 2006, Yeap *et al.*, 2014, Pye *et al.*, 2014, Antonio *et al.*, 2015). However, there is also a suggestion of reverse causality (i.e. that underlying ill health causes a reduction in testosterone levels) (Eriksson *et al.*, 2017). For example, Laaksonen *et al.* (2005) suggested that presence of the metabolic syndrome predisposes middle-aged men to the development of hypogonadism. However, serum gonadotrophins were not measured so the existence of subclinical hypogonadism at baseline could not be ruled out.

The causal relationship between ageing, testosterone and disease is clearly complex and requires further clarification. Nevertheless, androgen replacement therapy is becoming increasingly common with the number of testosterone prescriptions increasing by 90% between 2001 and 2010 in the United Kingdom, costing the National Health Service an estimated 11.7 million pounds per year (Gan *et al.*, 2013). Similar increasing trends in testosterone prescribing are also reported in the United States (Baillargeon *et al.*, 2013) and Australia (Handelsman, 2012) despite conflicting evidence regarding the risks and benefits of androgen replacement therapy. Some studies have reported beneficial effects of androgen replacement therapy on muscle, lipid and glucose metabolism in hypogonadal men (Kovacheva *et al.*, 2010, Frederiksen *et al.*, 2012, Yassin *et al.*, 2016, Sharma *et al.*, 2017). Conversely, Basaria *et al.* (2015) reported no beneficial effect of testosterone replacement on the progression of subclinical atherosclerosis in ageing men; but reported an increase in haematocrit and levels of prostate-specific antigen which may be indicative of an increased risk of adverse cardiovascular events and prostate cancer respectively (Osterberg *et al.*, 2014). Indeed, a clinical trial of testosterone replacement in elderly men was prematurely halted due to an increased frequency of adverse cardiovascular events in the testosterone treated group (Basaria *et al.*, 2010).

1.5 Testicular Ageing

1.5.1 Age-related Testicular Atrophy

In contrast to the female menopause, ageing in males is not accompanied by a cessation of reproductive capacity. However, as with many tissues, testicular atrophy is associated with advancing age although the age of onset and severity is highly variable between individuals. Handelsman and Staraj (1985) reported that testis size is reduced in aged males, independently of underlying ill health. More recently, scrotal ultrasound examination of 150 males up to 96 years of age revealed that maximum testis volume is seen in 25 year-old men and a slight but significant reduction is observed in ageing men (Well *et al.*, 2007). Similar findings were also reported by Yang *et al.* (2011). In addition, Mahmoud *et al.* (2003) related testicular volume to circulating hormone levels and demonstrated an indirect correlation between testicular volume and both luteinising hormone and follicle-stimulating hormone in ageing men. This suggests

that age-related testicular atrophy is not likely due to hypothalamic-pituitary failure and subsequent diminished testicular gonadotrophin stimulation. In contrast, Pasqualotto *et al.* (2005) did not detect an association between age and testicular volume. However, this study only included males up to 67 years of age. The authors did however report an increase in circulating FSH and decrease in semen sperm concentration in older men, consistent with age-related Sertoli cell dysfunction (Neaves *et al.*, 1984, Tenover *et al.*, 1988, Johnson *et al.*, 1990, Mahmoud *et al.*, 2000).

Several studies have suggested that the number of Sertoli cells declines as men age (Johnson *et al.*, 1984b, Jiang *et al.*, 2013, Petersen *et al.*, 2015), and that sperm output of the ageing testis is compromised (Chen *et al.*, 2003, Eskenazi *et al.*, 2003, Ng *et al.*, 2004, Johnson *et al.*, 2015). Ultrastructural studies have revealed progressive degeneration and involution of the seminiferous tubules associated with multinucleated germ cells, germ cell exfoliation and subsequent vacuolation of the Sertoli cells in the ageing testis (Paniagua *et al.*, 1987, Paniagua *et al.*, 1991, Jiang *et al.*, 2013). Additionally, the basement membrane of tubules with spermatogenic arrest is reported to be thickened (Paniagua *et al.*, 1987, Dakouane *et al.*, 2005, Sasano and Ichijo, 1969). Paniagua *et al.* (1987) suggested that these changes were similar to those induced by experimental testicular ischaemia which would be consistent with a reduction in capillary network in regions of tubule degeneration in aged testes (Suoranta, 1971). In addition, testicular atrophy and a reduction in sperm production is observed in the atherosclerotic ApoE$^{-/-}$;LDL receptor$^{-/-}$ double knockout mouse model in which testicular vascular volume is reduced (Langheinrich *et al.*, 2012). Interestingly, Sertoli cell function has been suggested to modulate the vascular network in the mouse testis (Rebourcet *et al.*, 2016) and, the testicular vasculature has been reported to contribute to the spermatogonial stem cell niche (Yoshida *et al.*, 2007). As such, the interdependence between ageing, the testicular microvasculature and seminiferous tubule function is complex and the sequence of events leading to testicular atrophy/seminiferous tubule degeneration is not fully understood. In general, the testes of aged men contain a mosaic of seminiferous tubules with apparently normal spermatogenesis as well as completely atrophic tubules and variation between individuals is high, probably explaining the individual variation in testis volume.

There are conflicting reports on the fate of the Leydig cell population during the ageing process. Kothari and Gupta (1974) initially reported that Leydig cell number was in fact increased in the ageing testis. However, their analysis was based only on total Leydig cell volume per testis. Thus, the observed increase may be due to Leydig cell hypertrophy rather than hyperplasia. In a later study, Kaler and Neaves (1978) reported that individual Leydig cell volume did not change significantly with advancing age but total Leydig cell volume and thus absolute Leydig cell number decreased with age. In another study, a forty-four percent reduction in Leydig cell number was observed between men aged 20-48 and 50-70 years old (Neaves *et al.*, 1984). Interestingly, the authors reported that this was not associated with a reduction in circulating testosterone, instead a two-fold elevation in luteinising hormone was observed in the older men (Neaves *et al.*, 1984). Contrary to these early reports, more recent evidence suggests that Leydig cell numbers do not decrease with age in men. Initially reporting the results of a pilot study, Petersen and Pakkenberg (2000) suggested that, whilst Sertoli cell number may decline with age, no such change is observed in the Leydig cell population. Using a larger sample size of men ranging from 16 to 80 years old, the authors later reported that an inverse relationship exists between cell number and advancing age for Sertoli cells but not for Leydig cells (Petersen *et al.*, 2015). It must be considered however, that each of the aforementioned studies quantifying Sertoli cell and Leydig cell numbers is cross-sectional. As such, it is not entirely possible to conclude whether cell number decreases due to ageing *per se* or due to a secular effect. Nevertheless, hormonal parameters (i.e. increased gonadotrophins) suggest that even if Sertoli and Leydig cell numbers do not decline with age, cell function may be reduced.

1.5.2 Testicular Endocrine Function Declines with Age

In humans, circulating testosterone is either specifically bound to sex hormone binding globulin (SHBG), weakly bound to albumin, or is not associated with binding proteins (i.e. is ‘free’) (Vermeulen and Verdonck, 1968, Kaufman and Vermeulen, 2005). Sex hormone binding globulin is produced by the liver whereas in rodents, the equivalent (androgen binding protein; ABP) is produced by testicular Sertoli cells (Joseph *et al.*, 1997). The ‘free’ and albumin-bound fractions are generally considered to represent bioavailable testosterone that is readily available to target tissues (Manni *et al.*, 1985).

However, the relative contribution of albumin-bound testosterone to the bioavailable pool is likely influenced by multiple factors in different tissue contexts (Vermeulen *et al.*, 1999). A consensus regarding the 'normal' reference range for testosterone has been lacking (Giannetta *et al.*, 2012). In a recent study, Travison *et al.* (2017) obtained serum samples from four large cohort studies previously conducted in the United States and Europe. Testosterone was re-measured in a subset of samples from each cohort to allow cross-calibration of the different assays previously used to measure testosterone in each of the cohorts. The authors then compared the newly measured values to those previously obtained to develop a normalisation strategy that they then applied to the entire cohorts. Using this strategy, normal reference range for testosterone in healthy men aged 19 to 30 years old is reported to be 264-916 ng/dL (Travison *et al.*, 2017).

As discussed in section 1.4.4, androgens are thought to support general health in men. Several longitudinal studies from Europe (Wu *et al.*, 2008), the United States (Harman *et al.*, 2001, Feldman *et al.*, 2002, Mohr *et al.*, 2005, Fabbri *et al.*, 2016) and Australia (Liu *et al.*, 2007) have demonstrated a decrease in testosterone and increase in SHBG (ultimately indicating decreased bioavailable testosterone) as males age, although the rate of decline varies between studies. Generally, testosterone decreases by 1-2% per year from the age of 40 years onwards. However, the presence of a clinical syndrome in which low testosterone is associated with adverse sexual, physical and psychological symptoms only manifests in a small number of aged males (Huhtaniemi, 2014). Intriguingly, it has been suggested that a greater age-related decline in testosterone is associated with later year of birth (Andersson *et al.*, 2007, Travison *et al.*, 2007, Perheentupa *et al.*, 2013, Mazur *et al.*, 2013). Although the mechanism(s) underlying the age-associated decline in testosterone and its clinical significance are not fully understood, the suggestion of a secular decline may indicate environmental/lifestyle factors are be of etiological importance.

Reduced levels of circulating testosterone (hypogonadism) can be due to either a defect in Leydig cell androgen biosynthesis (termed primary hypogonadism), or due to reduced stimulation of Leydig cells due to defects in the hypothalamic-pituitary unit (see section 1.3) resulting in decreased luteinising hormone secretion (termed secondary hypogonadism). Secondary hypogonadism is commonly associated with

obesity due to decreased luteinising hormone production (Vermeulen *et al.*, 1993) and its prevalence is not reported to increase with age Tajar *et al.* (2010). Conversely, the proportion of men with primary hypogonadism is increased with advancing age thus, age-related reduction in testosterone level is thought to be predominantly caused by primary testicular dysfunction (Tajar *et al.*, 2010, Tajar *et al.*, 2012).

Androgen levels may remain within the normal range in the face of Leydig cell dysfunction. In this scenario, increased pituitary-derived luteinising hormone is required to drive Leydig cell steroidogenesis (Rubens *et al.*, 1974, Andersson *et al.*, 2004, Tajar *et al.*, 2010). Given this plasticity in the hypothalamic-pituitary-gonadal axis, inferences of Leydig cell function can only be made from the luteinising hormone/testosterone ratio, with a high ratio indicative of Leydig cell dysfunction (compensated Leydig cell failure). It has been reported that the proportion of men with compensated Leydig cell failure also increases with age and is associated with an increased likelihood of physical symptoms considered to be related to low testosterone (including inability to carry out vigorous activity, walk more than one kilometre, or reduced flexibility)(Tajar *et al.*, 2010). Thus, it is suggested that compensated Leydig cell failure is a distinct clinical state associated with ageing and that men with compensated hypogonadism may be poised to develop primary hypogonadism as they age. As such, understating the mechanism(s) underlying age-related Leydig cell dysfunction is important to aid the development of novel therapeutic interventions aimed at reversing age-related primary hypogonadism.

1.5.3 Mechanisms of Reduced androgen Production

It has been suggested that aged Leydig cells may be less sensitive to gonadotrophin stimulation resulting in reduced testosterone production (Longcope, 1973, Rubens *et al.*, 1974, Harman and Tsitouras, 1980, Veldhuis *et al.*, 2012). Interestingly, the studies of Longcope (1973) and Rubens *et al.* (1974) reported that the relative increase in gonadotrophin-stimulated testosterone production over baseline was similar between young and old men. This raises the possibility that a deficit in Leydig cell number, rather than function, may underlie the reduced ability of the aged testis to produce testosterone in this setting (i.e. when gonadotrophin level is limiting). Consistent with this theory, Neaves *et al.* (1984) suggested that increased luteinising hormone

production by the pituitary is able to maintain testosterone production in the testes of aged men which were reported to contain fewer Leydig cells. However, in light of the suggestion that Leydig cell number does not decline with age (Petersen *et al.*, 2015); decreased steroidogenic efficiency of aged Leydig cells may indeed be responsible for age-related primary hypogonadism. This is further supported by studies using the aged Brown Norway rat as a model of human reproductive ageing (in which Leydig cell numbers are not reported to decrease with age) that demonstrate a blunted response (i.e. reduced androgen biosynthesis) of aged Leydig cells to gonadotrophin stimulation (Chen *et al.*, 1994, Chen *et al.*, 2002).

1.5.3.1 Impaired Luteinising Hormone Receptor Signalling

Impaired luteinising hormone receptor (LHCGR) signalling (see section 1.4.1) has been described in aged Leydig cells. A reduction in LHCGR number on the surface of aged Leydig cells results in decreased intracellular cAMP accumulation following stimulation with luteinising hormone (Chen *et al.*, 2002). Stimulation of Leydig cells with forskolin (which raises cAMP independently of LHCGR signalling) causes equivocal increases in cAMP in young and aged cells; suggesting that adenylyl cyclase activity is maintained in aged cells (Chen *et al.*, 2002). Furthermore, incubation of aged Leydig cells with saturating concentrations of dibutyryl cAMP (a membrane-permeable cAMP agonist that bypasses the LHCGR/adenylyl cyclase cascade) restores testosterone production by aged cells to levels produced by young cells (Chen *et al.*, 2004). Together, these studies suggest that LHCGR signal transduction is less efficient in aged Leydig cells.

1.5.3.2 Defects in the Steroidogenic Cascade

A number of defects in the steroidogenic pathway have been identified in aged rat Leydig cells. Firstly, Culty *et al.* (2002) reported that cholesterol transport across the mitochondrial membrane is reduced, consistent with a reduction in mRNA and protein expression of StAR and TSPO in aged Leydig cells (Luo *et al.*, 2001, Culty *et al.*, 2002, Luo *et al.*, 2005). The contribution of the latter to the age-related reduction in androgen biosynthesis may be insignificant as recent evidence suggests that TSPO may be redundant for normal steroidogenesis (Morohaku *et al.*, 2014, Tu *et al.*, 2014). However, Chung *et al.* (2013) reported that pharmacological activation of TSPO both

in vitro and *in vivo* resulted in increased testosterone production by aged rat Leydig cells to levels equivalent of young controls. In addition to impaired cholesterol trafficking, mRNA and protein expression of CYP11A1, HSD3B and CYP17A1 is decreased in aged Leydig cells (Luo *et al.*, 1996, 2001, Culty *et al.*, 2002, Luo *et al.*, 2005). Furthermore, incubation of Leydig cells with either 25-hyroxycholesterol, pregnenolone, progesterone, 17-hydroxyprogesterone, or androstenedione, as substrates for androgen production, allowed individual reactions in the steroidogenic cascade to be monitored. This demonstrated that the enzymatic activity of CYP11A1, HSD3B, CYP17A1 and HSD17B are all reduced in aged Leydig cells (Luo *et al.*, 1996).

1.5.3.3 Pro/Antioxidant Balance & Cumulative Damage

Leydig cell turnover in the adult testis is thought to be minimal (Teerds *et al.*, 1989, Mendis-Handagama, 1991) thus, the terminally differentiated Leydig cell population, which is established during pubertal development, is probably not replenished to any significant extent. As such, the reduced capacity for androgen production by aged Leydig cells likely reflects a degenerative response to a myriad of insults received over the lifetime of individual cells. Initially proposed by Harman (1956), the 'free-radical theory of ageing' suggests that cumulative damage by reactive oxygen species (ROS) results in impaired cellular function which in turn leads to age-related tissue degeneration (Finkel and Holbrook, 2000). Reactive oxygen species are generated as a by-product of mitochondrial respiration (Turrens, 2003). As highly reactive molecules, they can induce damage to lipid membranes, proteins and DNA and interfere with intracellular signalling cascades. Gonadotrophin stimulation of primary rat Leydig cells and the MA-10 mouse Leydig cell tumour line has been shown to increase intracellular ROS concentrations and induce DNA damage, an effect which is more profound in aged Leydig cells (Beattie *et al.*, 2013).

In steroidogenic cells such as Leydig cells, ROS are also generated by cytochrome P450 enzymes which function in the steroidogenic cascade (Hanukoglu, 2006). As such, Leydig cells are equipped with enzymatic and non-enzymatic antioxidant defence systems to prevent ROS-induced damage (Aitken and Roman, 2008). During the ageing process, the oxidant/antioxidant balance is thought to become

compromised, leading to establishment of an oxidative environment that impairs cellular functions. Indeed using the redox-sensitive probe lucigenin, Chen *et al.* (2001) detected increased levels of superoxide in aged rat Leydig cells and it has been reported that the expression and activity of key antioxidant enzymes including superoxide dismutase-1 and -2, glutathione peroxidase as well as the antioxidant glutathione, is reduced in aged Leydig cells and testis tissue (Cao *et al.*, 2004, Luo *et al.*, 2006, Huang *et al.*, 2017). Furthermore, glutathione depletion both *in* vitro and *in vivo* reduces Leydig cell testosterone production in normal Leydig cells (Chen *et al.*, 2008).

Further evidence that steroidogenesis itself may cause cumulative damage to Leydig cells is provided by an experiment conducted by Chen and Zirkin (1999), in which long-term suppression of Leydig cell steroidogenesis *via* silastic testosterone implants to supress endogenous testosterone production, protects cells against age-related reductions in testosterone biosynthesis. Additionally, when hypo-functional aged Leydig cells are ablated from the rat testis using ethane dimethanesulphonate (EDS), the 'new' Leydig cells which repopulate the aged testis are able to restore testosterone to levels observed in young controls (Chen *et al.*, 1996). However, in a more recent study, Chen *et al.* (2015) assessed the fate of newly re-generated Leydig cells 10 and 30 weeks post EDS ablation and found that the 'new' Leydig cells in the aged testis failed to maintain their ability to produce testosterone compared to those of young animals. As such, factors extrinsic to Leydig cells in aged animals (i.e. within the testicular microenvironment or systemic factors) may also contribute to age-related Leydig cell dysfunction.

Despite the accumulation of knowledge regarding the age-related changes which occur in Leydig cells, the precise mechanism(s) which lead to such changes remain to be elucidated. Deeper mechanistic insights into the age-related dysfunction of Leydig cells could identify novel therapeutic strategies to promote androgen production in ageing males thus improving quality of life in older males as well as reducing the healthcare burden of an increasing aged population.

1.6 Animal Models for the Study of Testicular Ageing

1.6.1 Naturally Aged Animals

Naturally aged animals have been widely used to characterise the impact of ageing on the reproductive system. The naturally aged Brown Norway rat is perhaps the best studied; much of what is known about the age-related changes in the testis, particularly with reference to Leydig cell testosterone production (discussed in section 1.5.3), has arisen through studies conducted using this model. Importantly, compared to other strains of rat including Fisher F344 (Turek and Desjardins, 1979), Sprague-Dawley (Kaler and Neaves, 1981), and Wistar (Teerds *et al.*, 1991), in which circulating gonadotrophins are maintained or even decreased; the hormonal profile of aged Brown Norway rats is considered to best resemble the age-related primary hypogonadism observed in ageing men (i.e. increased LH and decreased testosterone)(Chen *et al.*, 1994). Studies of naturally ageing mice report conflicting findings regarding the impact of ageing on circulating testosterone. It has been suggested that LH production decreases with age leading to reduced circulating testosterone (Bronson and Desjardins, 1977, Coquelin and Desjardins, 1982). In contrast, Finch *et al.* (1977) reported no difference in LH and testosterone levels between young and old mice. As such, naturally aged mice may be of limited utility for the study of age-related Leydig cell dysfunction.

Intriguingly, Poutahidis *et al.* (2014) reported that consumption of probiotic microbes could promote spermatogenesis and steroidogenesis during ageing. Specifically, in outbred CD-1 mice, consumption of *Lactobacillus reuteri* resulted in increases in testis weight, seminiferous tubule cross-sectional area, germ cell volume, Leydig cell number and circulating testosterone (Poutahidis *et al.*, 2014). This suggests that the composition and/or health of the intestinal microflora may be an important mediator testicular function during the ageing process. The authors further suggested that anti-inflammatory properties of *Lactobacillus reuteri* may be responsible for the observed ‘anti-ageing’ effects on the testis as systemic administration of blocking antibodies against the pro-inflammatory cytokine interleukin-17 produced similar effects on the above reproductive parameters in 12-month old mice (Poutahidis *et al.*, 2014). Unfortunately, circulating gonadotrophins were not measured in these studies, so

whether the ‘anti-aging’ effects of both *Lactobacillus reuteri* and interleukin-17 blockade act at the hypothalamic/pituitary level or directly on the testis remains to be established.

1.6.2 Premature Ageing Models

Studying naturally aged animals is time consuming, expensive and is accompanied by protracted welfare issues. As such, models of premature ageing may be attractive alternatives for the expedited study of age-related testicular dysfunction. However, whilst prematurely ageing models may display general degenerative phenotypes resembling ageing, they may not fully recapitulate normal ageing processes. As such, the characteristics of individual models must be carefully considered in order to select the most appropriate model for use in tissue/organ-specific ageing research projects. For example, administration of D-galactose to mice and rats results in accelerated ageing and is reported to model aspects of age-related primary hypogonadism. D-galactose treatment induces oxidative stress in the testis and is associated with decreased testis weight, reduced sperm production, decreased circulating testosterone and an increase in both LH and FSH (Ahangarpour *et al.*, 2014, Liao *et al.*, 2016, Yang *et al.*, 2015a). Additionally, in the senescence accelerated SAMP8 mouse line, premature ageing is also thought to be partly mediated by oxidative stress. SAMP8 mice have increased levels of DNA damage in sperm as well as decreased circulating testosterone in the presence of increased gonadotrophins - indicative of primary testicular dysfunction (Flood *et al.*, 1995, Smith *et al.*, 2013, Wang *et al.*, 2016a). Conversely, Trifunovic *et al.* (2004) described an accelerated ageing phenotype characterised by weight loss, hair loss, osteoporosis and premature death in mitochondrial DNA (mtDNA) mutator mice that lack the proofreading activity of mitochondrial DNA polymerase. A significant reduction in testis weight and reduced fertility, associated with degeneration of the seminiferous epithelium concomitant with increased FSH is observed in mtDNA mutator mice (Trifunovic *et al.*, 2004, Shabalina *et al.*, 2015). However, whilst Leydig cells of mtDNA mutator mice contain dysfunctional mitochondria and show signs of oxidative stress, circulating testosterone and LH are normal (Shabalina *et al.*, 2015).

1.6.2.1 The CISD2-Deficient Prematurely Ageing Model

In 2009, a premature ageing phenotype characterised by nerve and muscle degeneration, osteopenia, reduced body weight and premature death, was reported in mice deficient for *Cisd2* (CDGSH iron sulphur domain 2), (Chen *et al.*, 2009c). Conversely, transgenic *Cisd2* over expression in mice has been reported to extend healthy lifespan and delay ageing; protecting against age-associated mitochondrial dysfunction (Wu *et al.*, 2012). Interestingly, human *CISD2* is located on chromosome 4q, in a region that has been associated with longevity (Puca *et al.*, 2001).

Cisd2 encodes the second member of small family of transmembrane proteins that contain a CDGSH iron sulphur domain. There is little known regarding the regulation and function of CISD2, even the subcellular localisation is disputed with conflicting reports suggesting it is localised to the mitochondrion (Chen *et al.*, 2009c) and/or the endoplasmic reticulum (Chang *et al.*, 2010, Wiley *et al.*, 2013). Chang *et al.* (2010) suggested that CISD2 may be involved in facilitating the interaction of BCL-2 and beclin-1, an interaction that antagonises the autophagy pathway. It has also been suggested that CISD2 may be involved in the cellular response to oxidative stress (Tamir *et al.*, 2013, Tamir *et al.*, 2015). Indeed, using a redox-sensitive fluorescent probe, Wiley *et al.* (2013) demonstrated that CISD2-loss results in a pro-oxidative intracellular environment. Furthermore, CISD2-loss causes endoplasmic reticulum stress and mitochondrial dysfunction associated with dysregulation of intracellular calcium homeostasis (Chang *et al.*, 2010, Chang *et al.*, 2012, Wiley *et al.*, 2013, Wang *et al.*, 2014).

The reproductive phenotype of CISD2-deficient mice has not been characterised. As such, whether this prematurely ageing model has utility for the studies of age-related testicular dysfunction remains to be established. In humans, *CISD2* mutation is associated with Wolfram syndrome, which is characterised by early onset of age-associated phenotypes including juvenile-onset diabetes, progressive neurologic degeneration, and endocrine dysfunction (Amr *et al.*, 2007, Rohayem *et al.*, 2011). Testicular atrophy and infertility have also been described in patients with Wolfram syndrome. More specifically, reduced testosterone accompanied by elevated gonadotrophins (indicative of primary testicular dysfunction) has been reported (Peden

et al., 1986, Homan and MacKay, 1987, Barrett *et al.*, 1995, Medlej *et al.*, 2004, Rohayem *et al.*, 2011, Haghighi *et al.*, 2013). This indirect evidence suggests that *Cisd2*-deficient mice may be expected to model age-related primary hypogonadism as observed in ageing men.

1.7 Therapeutic Potential of Stem Cells to treat Primary Hypogonadism

As discussed in section 1.4.4, hypogonadism (i.e. in the ageing male) is associated with an increased risk of chronic, age-related cardiometabolic disorders. Age related hypogonadism is thought to be partly due to primary testicular dysfunction (see section 1.5.2). Indeed, a number of defects, which may contribute to reduced testosterone production, have been described in aged Leydig cells (discussed in section 1.5.3). Exogenous testosterone replacement is widely used to treat hypogonadism although the risks and benefits associated with the administration of exogenous testosterone are not entirely clear (Bassil *et al.*, 2009, Handelsman, 2017). As an alternative to exogenous androgen replacement, recent efforts have been made to develop stem cell-based therapies to treat testicular dysfunction.

In general, there have been two approaches towards a stem cell therapy for the treatment of primary hypogonadism. Firstly, significant attention has been focussed on the isolation and *in vitro* characterisation of endogenous stem Leydig cells and their subsequent transplantation into the testis. An initial study suggested that a 'side population' of cells isolated from the mouse testis contained stem/progenitor cells that could form Leydig cells and increase testosterone levels when transplanted into hypogonadal luteinising hormone receptor-deficient mice (Lo *et al.*, 2004). However, the precise identity of the responsible stem cell population was unknown. As mentioned in section 1.2.1.1.1, Ge *et al.* (2006) isolated a population of stem cells from the neonatal rat testis based on expression of platelet-derived growth factor receptor-alpha. The authors suggested these cells could differentiate into testosterone producing Leydig-like cells *in vitro* when cultured in the presence of a differentiation inducing medium containing luteinising hormone, insulin-like growth factor 1, platelet-derived growth factor-beta and thyroid hormone. This cocktail differentiation inducing factors was also reported to induce Leydig cell differentiation of nestin-positive (Jiang *et al.*, 2014) and CD51-positive (Zang *et al.*, 2017) testicular stem cells isolated from the

mouse testis. Whilst these studies provide promising evidence that endogenous stem cells may have therapeutic potential for the treatment of primary hypogonadism, isolation of stem cells from a patient's testis would be impractical, as the testis is digested to isolate the cells. Theoretically, activation of endogenous stem Leydig cells would circumvent the need for cell isolation and transplantation procedures however, the factors controlling stem Leydig cell fate are yet to be fully elucidated.

The second approach involves the use of extra-gonadal stem cells, including pluripotent stem cells (i.e. embryonic or induced pluripotent stem cells) and multipotent mesenchymal stem cells (MSCs). For example, Yang *et al.* (2015b) reported that murine embryonic stem cells could be transformed into Leydig-like cells *via* steroidogenic factor-1 (SF-1) overexpression and, when transplanted into Leydig cell ablated rat testes, could transform into mature Leydig cells. More recently, to overcome ethical and safety concerns concomitant with the use of pluripotent embryonic stem cells, a protocol to transform mouse fibroblasts into Leydig cells *via* viral transduction of SF-1, DMRT-1 and GATA-4 was described (Yang *et al.*, 2017b). The authors demonstrated that forced expression of these transcription factors resulted in the reprogramming of fibroblasts into Leydig cells, without the induction of a pluripotent state. However, this procedure was more efficient in embryonic fibroblasts compared to fibroblasts isolated from adult tissue.

In the case of mesenchymal stem cells, bone marrow MSCs have been reported to differentiate into Leydig cells when transplanted into the mouse testis (Yazawa *et al.*, 2006, Lue *et al.*, 2007). However, further *in vitro* characterisation of steroidogenic cells derived from bone marrow MSCs suggested they might favour glucocorticoid over androgen production (Yazawa *et al.*, 2006). Transfection of adipose-derived MSCs with SF-1 is reported to induce their differentiation into steroidogenic cells *in vitro*. However, similar to the aforementioned reports of bone marrow MSCs, the resulting cells appear to predominantly produce glucocorticoids (Gondo *et al.*, 2008). Adipose-derived mesenchymal stem cells have been reported to improve Leydig cell function in an experimentally induced ageing model although the positive effects may be indirect *via* suppression of reactive oxygen species rather than differentiation into Leydig cells (Yang *et al.*, 2015a). Similarly, Zhang *et al.* (2017) suggested that

umbilical cord MSCs could promote Leydig cell regeneration following EDS-mediated ablation, but very few of the transplanted cells appeared to form Leydig cells.

1.8 Hypothesis, Aims & Approach

As outlined in the preceding sections, the mammalian testis is a complex organ divided into two distinct compartments that carry out its principal functions of spermatogenesis and steroidogenesis. Both these processes are reported to become impaired as males age. Perhaps of most significance is the reported age-related decrease in androgen production as androgens are thought to support lifelong general health in men. It is well established that testicular function is critically dependent on the somatic Sertoli and Leydig cells and age-related degenerative changes have been described in each of these cell populations. However, the interrelationship between testicular function, ageing and disease is not fully understood. Specifically, the paracrine interactions between testicular cell populations that support testicular function and the extent to which intrinsic age-related Sertoli and Leydig dysfunction contributes to alterations in the testicular microenvironment remain to be established.

1.8.1 Hypothesis

The main hypothesis underpinning the experiments described herein is that manipulation of the paracrine network in the testis *in vivo* would provide significant novel insights into the regulation of testicular function, uncovering potential therapeutic targets to treat age-related testicular dysfunction.

1.8.2 Aims

1. To determine the utility of novel premature ageing mouse models for the expedited study of testicular ageing and to define the contribution of Leydig and Sertoli cells to age-related primary hypogonadism.
2. To establish the role(s) of leukemia inhibitory factor as a putative paracrine regulator of testicular development and function *in vivo.*
3. To determine the regenerative potential of human perivascular stem cells to promote endogenous testosterone production as a potential therapeutic strategy to treat hypogonadism.

1.8.3 Approach

To address the first aim, a novel *Cisd2* premature ageing allele was utilised. In the first instance, the testicular phenotype of constitutive *Cisd2*-deficient mice was characterised and compared to that of naturally aged mice. Next, the conditional potential of the novel *Cisd2* allele was exploited to limit premature ageing to Leydig cells or Sertoli cells separately such that the impact of ageing in these cell populations on testicular function could be determined.

To dissect out and characterise the role of leukemia inhibitory factor signalling in the testis, novel models of testis cell-specific LIFR ablation were generated and characterised to determine the influence of paracrine LIF/LIFR signalling in the regulation of testicular development and function.

To address the third aim, the ability of human adipose-derived perivascular stem cells to differentiate into Leydig cells under defined conditions *in vitro* was determined. In addition, a model of Leydig cell ablation and regeneration was used to establish the impact of human adipose-derived perivascular stem cells on Leydig cell regeneration *in vivo*.

2 Materials & Methods

2.1 Animals

2.1.1 Husbandry, Welfare and Ethics

All animal experiments were carried out in the Biomedical Research Facility Little France (LF2) in strict compliance with the Animals (Scientific Procedures) Act, 1986. All procedures were conducted in accordance with UK Home Office regulations under project licences 60/4200 and 70/8804 held by Professor Lee B. Smith. Animals were maintained on a 12hr light cycle (07:00 – 19:00) with controlled temperature (20-25^{o}C) and humidity (~55%). Soya-free chow and drinking water were available *ad libitum.* Daily husbandry and technical assistance with experimental procedures were carried out by Mr Michael Dodds (mice) and Mr William Mungall (rats) – Senior Technical Officers within Central Bioresearch Services.

2.1.2 Transgenic Mouse Lines

Both constitutive and conditional knockout alleles used in the studies herein were obtained from the International Mouse Phenotyping Consortium (IMPC; http://www.mousephenotype.org/). The majority of embryonic stem cell and/or mouse lines produced by the IMPC harbour the 'knockout-first' allele (Figure 2.1). These alleles are designed around a critical exon, shared between all transcript variants which, upon removal, results in a frame-shift mutation (theoretically) abolishing protein expression. While knockout-first alleles are targeted, they essentially function as gene-trap knockouts in the first instance as the large targeting cassette disrupts gene expression. Owing to the elegant design of the targeting cassette, (Testa *et al.*, 2004, Skarnes *et al.*, 2011), targeted mutation-1a alleles (*Tm1a*; Figure 2.1) are highly versatile and, upon exposure to site-specific recombinases, can give rise to reporter-tagged knockout (*Tm1b*), conditional (*Tm1c*), and null (*Tm1d*) alleles (Figure 2.1).

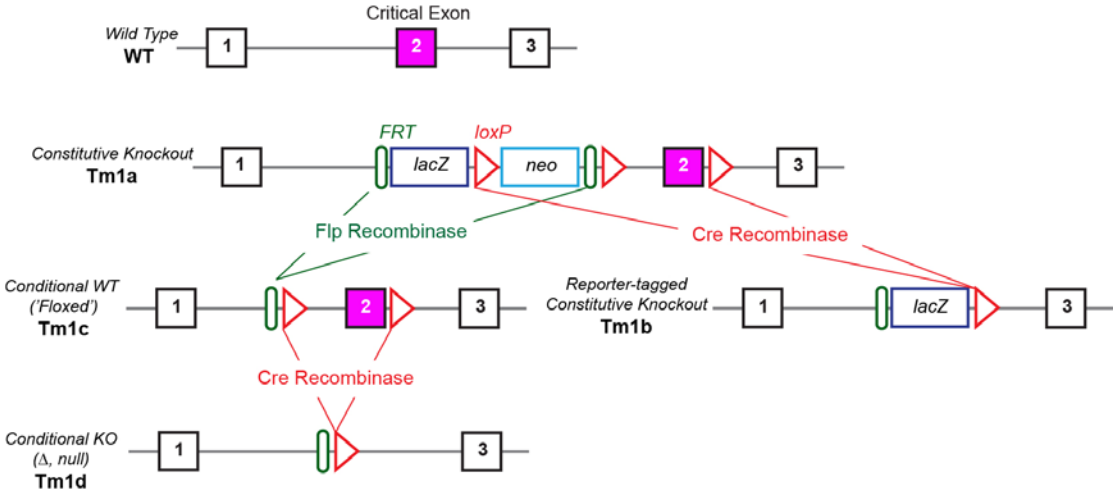


Figure 2.1 Schematic of the Knockout-First Allele. To generate the targeted mutation 1a (*Tm1a*) allele, the wild-type allele is targeted in C57BL/6N embryonic stem cells, through homologous recombination mediated by 5' and 3' homology arms of the targeting vector. The *Tm1a* allele can be converted to a reporter-tagged knockout allele (*Tm1b*) or a conditional allele (*Tm1c*) by exposure to Cre or Flp recombinase respectively. The *Tm1c* allele can be further converted to a null allele (*Tm1d*) upon exposure to Cre recombinase.

2.1.2.1 Constitutive Knockout Alleles

2.1.2.1.1 Constitutive *Cisd2*-knockout

Mice carrying a knockout-first *Cisd2* allele (*Cisd2*$^{tm1a(EUCOMM)Wtsi}$) were obtained from the IMPC project. Constitutive *Cisd2*-knockout mice were generated by crossing male and female *Cisd2*$^{wt/tm1a}$ mice to generate *Cisd2*$^{wt/wt}$, *Cisd2*$^{wt/tm1a}$ and *Cisd2*$^{tm1a/tm1a}$ offspring. Animals were genotyped as described in section 2.1.2.6, using the appropriate primers listed in Table 2-1. The genotyping strategy is outlined in Figure 2.2.

2.1.2.1.2 Constitutive *Lifr*-knockout

Mice carrying a reporter-tagged *Lifr* knockout allele (*Lifr*$^{tm1b(EUCOMM)Hmgu}$) were obtained from the IMPC project. Constitutive *Lifr*-knockout mice were generated by crossing male and female *Lifr*$^{wt/tm1b}$ mice to generate *Lifr*$^{wt/wt}$, *Lifr*$^{wt/tm1b}$ and *Lifr*$^{tm1b/tm1b}$ offspring. Animals were genotyped as described in section 2.1.2.6, using the appropriate primers listed in Table 2-1. The genotyping strategy is outlined in Figure 2.2.

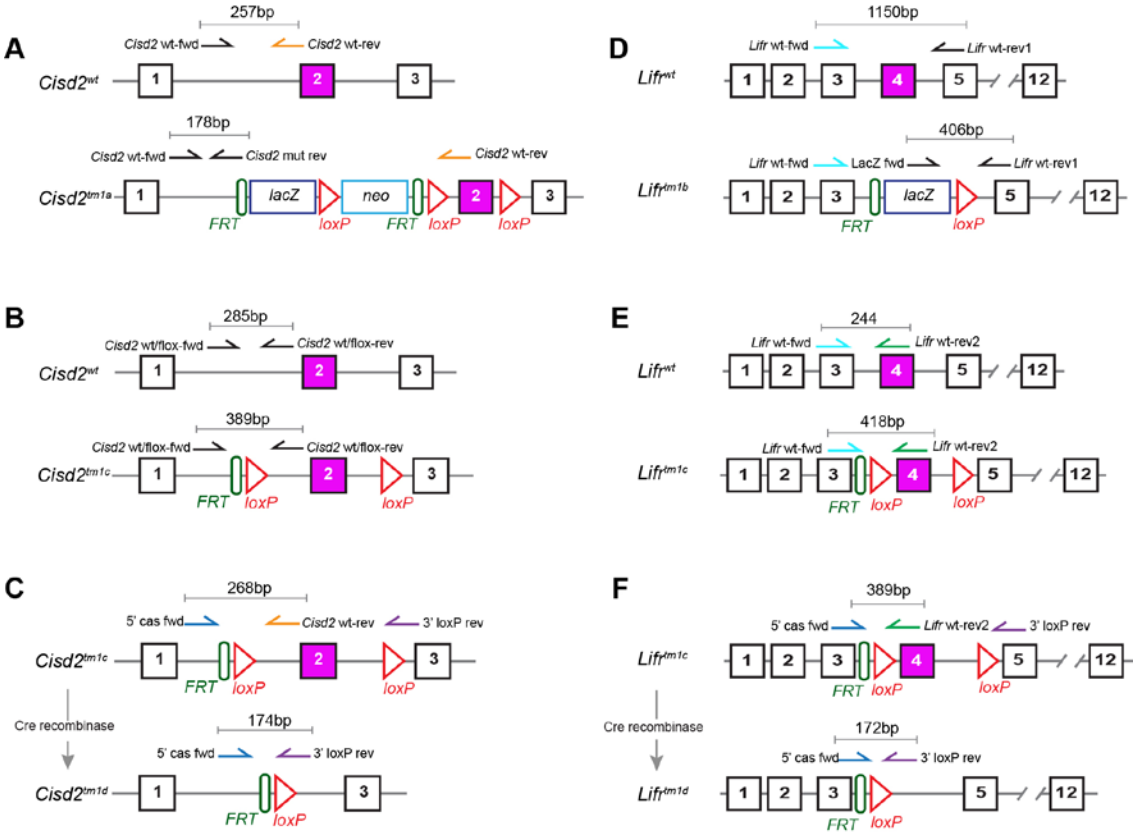


Figure 2.2 Constitutive and Conditional Alleles. Schematic representation of constitutive (*tm1a*) and conditional (*tm1c*) *Cisd2* (**A** and **B**) and *Lifr* (**D** and **E**) alleles. The location of genotyping primers are highlighted. Primer sequences and further details are listed in Table 2-1. Conversion of conditional *Cisd2* and *Lifr* alleles to null (tm1d) alleles following Cre exposure is illustrated in (**C**) and (**F**) respectively.

2.1.2.2 Conditional Knockout Alleles

While constitutive knockout alleles (e.g. *Tm1a* and *Tm1b*) are powerful tools for interrogating gene function, determining specific tissue/cell contributions to any observed phenotype becomes challenging. Furthermore, constitutive gene deletion may result in lethal developmental phenotypes, making it difficult, if not impossible, to study tissue/cell specific gene function during postnatal life. The Cre/*LoxP* system of conditional gene deletion permits spatio-temporal control of gene deletion (Nagy, 2000). Briefly, the Cre/*LoxP* system relies on the cyclisation recombination (Cre) enzyme from bacteriophage P1 which recognises and induces recombination between two *LoxP* sites engineered around a critical portion of the target gene sequence (Figure 2.1). The use of a cell/tissue-specific promoter to drive Cre expression limits recombination to the cell/tissue type of interest.

2.1.2.2.1 Conditional *Cisd2*-knockout

Mice carrying a conditional *Cisd2* allele (*Cisd2*$^{tm1c(EUCOMM)Wtsi}$) were obtained from the IMPC project. Cell-specific *Cisd2*-knockout mice were generated by mating *Cisd2*^{tm1c} mice to Cre recombinase-expressing mice (see section 2.1.2.3). Animals were genotyped as described in section 2.1.2.6, using the appropriate primers listed in Table 2-1. The genotyping strategy is outlined in Figure 2.2.

2.1.2.2.2 Conditional *Lifr*-knockout

Mice carrying a conditional *Lifr* allele (*Lifr*$^{tm1c(EUCOMM)Hmgu}$) were obtained from the IMPC project. Cell-specific *Lifr*-knockout mice were generated by mating *Lifr*^{tm1c} mice to Cre recombinase-expressing mice (see section 2.1.2.3). Animals were genotyped as described in section 2.1.2.6, using the appropriate primers listed in Table 2-1. The genotyping strategy is outlined in Figure 2.2.

2.1.2.3 Cre Recombinase Alleles

As outlined above, conditional gene deletion relies on tissue/cell-specific Cre expression. Several Cre-expressing mouse lines were used in these studies to convert conditional (*Tm1c*) alleles to null (*Tm1d*) alleles in specific testicular cell populations.

2.1.2.3.1 Leydig Cell Conditional Knockouts

Conversion of conditional (*Tm1c*) to null (*Tm1d*) alleles within testicular Leydig cells was achieved using a mouse line expressing Cre-recombinase under control of the Platelet-Derived Growth Factor Receptor-beta (*Pdgfrb*) promoter. *Tg(Pdgfrb-cre)*^{9Rha} mice (Foo *et al.*, 2006) were gratefully received from Professor Neil Henderson (MRC Centre for Inflammation Research, University of Edinburgh). Animals were genotyped as described in section 2.1.2.6, using the appropriate primers listed in Table 2-1.

2.1.2.3.2 Sertoli Cell Conditional Knockouts

Conversion of conditional (*Tm1c*) to null (*Tm1d*) alleles within testicular Sertoli cells was achieved using a mouse line expressing Cre-recombinase under the control of the Anti-Müllerian Hormone (*Amh*) promoter. *Tg(AMH-cre)*^{1Flor} mice (Lecureuil *et al.*, 2002) were obtained from a colony maintained on site by Professor Lee Smith (MRC Centre for Reproductive Health, University of Edinburgh). Animals were genotyped as described in section 2.1.2.6, using the appropriate primers listed in Table 2-1.

2.1.2.3.3 Germ Cell Conditional Knockouts

Conversion of conditional (*Tm1c*) to null (*Tm1d*) alleles within testicular germ cells was achieved using a mouse line expressing Cre recombinase under the control of the Stimulated by Retinoic Acid-8 (*Stra8*) promoter. *Tg(Stra8-icre)*^{1Reb} mice (Sadate-Ngatchou *et al.*, 2008) were obtained from a colony maintained on site by Professor Lee Smith (MRC Centre for Reproductive Health, University of Edinburgh). Animals were genotyped as described in section 2.1.2.6, using the appropriate primers listed in Table 2-1.

2.1.2.4 Cre Recombinase Reporter Alleles

In order to determine the utility of Cre-expressing lines for the targeting of specific cell populations within the testis, mice carrying Cre-inducible reporter alleles were used. Briefly, these alleles consist of transgenes, expressed from the ubiquitous *Rosa26* locus in *Gt(ROSA)*^{26Sor} mice, encoding fluorescent proteins downstream of a *LoxP*-flanked STOP codon. Following Cre exposure, the STOP codon is removed resulting in expression of the fluorescent protein.

2.1.2.4.1 Red Fluorescent Protein Cre-reporter Mice

Mice carrying the red fluorescent protein (RFP) reporter allele *Gt(ROSA)*$^{26Sortm14(CAG-tdTomato)Hze}$ (Madisen *et al.*, 2010) were gratefully received from Professor Neil Henderson (MRC Centre for Inflammation Research, University of Edinburgh). Animals were genotyped as described in section 2.1.2.6, using the appropriate primers listed in Table 2-1.

2.1.2.5 Timed Matings

To obtain embryos at specific gestational time points, timed matings were conducted. Male and female breeding animals were paired in the afternoon and females were monitored for copulatory plugs the following morning. The day of plug discovery was designated embryonic (e) day 0.5.

2.1.2.6 Genotyping

Inheritance of transgenes in offspring derived from transgenic matings was determined by transgene-specific polymerase chain reaction (PCR) assays. Routine genotyping was performed by the technical support team in the laboratory of Professor Lee Smith.

I am particularly grateful to Laura Milne, Sarah Smith, Nathan Jeffery and Lyndsey Wilson (née Cruickshanks) for assistance with genotyping.

2.1.2.6.1 Genomic DNA Extraction

Genomic DNA (gDNA) was extracted from ear biopsies taken at weaning. Briefly, ear biopsies were digested in 25uL TE-Tween (section 2.9.1.3) supplemented with 20µg of proteinase K (Sigma-Aldrich, Dorset, UK) for 1 hour at 55ºC. Samples were then incubated at 95ºC for 7mins to denature remaining proteinase K before being cooled to 25ºC. Incubations were carried out on a Tprofessional thermocycler (Biometra GmbH, Göttingen, Germany). Following digestion, samples were briefly centrifuged at 2500rpm to pellet undigested tissue. The supernatant was diluted 1:10 in sterile water for use in downstream PCR assays. Samples were stored at 4ºC short term, or -20ºC long term.

2.1.2.6.2 Genomic PCR

PCR was performed on crude gDNA extracts (section 2.1.2.6.1) using either BioMix™ PCR reaction buffer (Bioline Reagents Ltd, London, UK) or Type-it Mutation Detect PCR Kit (QIAGEN Ltd., Crawley, UK) according to the manufacturer's instructions. Details of individual assays including primer sequences, annealing temperatures and PCR buffer are listed in Table 2-1. PCR products were analysed using the QIAxcel capillary electrophoresis system (QIAGEN Ltd., Crawley, UK). A reaction containing no template (NTC) was included to ensure reagents were free from contaminating nucleic acids.

Table 2-1. Details of Primers used for Genotyping Assays

Assay	Forward Primer(s)	Reverse Primer(s)	Annealing Temp. (°C)	Master Mix	Product Size (bp)
Cisd2^{tm1a}	Cisd2 wt-fwd GAATATTCAATGTGTAAAGGTTTCAA	Cisd2 wt-rev GAAAACATTTTCACTCCTTTCTTTTT Cisd2 mut-rev GAACTTCGGAATAGGAACTTCG	56	Biomix	*Cisd2*^{wt} - 257 *Cisd2*^{tm1a} - *178*
Cisd2^{tm1c}	Cisd2 wt/flox-fwd GAGTAAGTTGACTGATCAGG	Cisd2 wt/flox-rev CCTATCTCATTAGATCTGCT	56	Type-it	*Cisd2*^{wt} - 285 *Cisd2*^{tm1c} - 389
Cisd2^{tm1d}	5'cas-fwd AAGGCGCATAACGATACCAC	Cisd2 wt-rev GAAAACATTTTCACTCCTTTCTTTTT 3' loxP-rev ACTGATGGCGAGCTCAGACC	61	Type-it	*Cisd2*^{tm1c} - 268 *Cisd2*^{tm1d} - 174
Lifr^{tm1b}	Lifr wt-fwd GGAAACCCTGGTATTGTGGA LacZ-fwd CCAGTTGGTCTGGTGTCA	Lifr wt-rev CATGCCACAGTGCGACAG	59	Type-it	*Lifr*^{wt} - 1150 *Lifr*^{tm1b} - 406
Lifr^{tm1c}	Lifr wt-fwd GGAAACCCTGGTATTGTGGA	Lifr wt-rev2 GGCTGTCCTGGAACTCACTC	59	Type-it	*Lifr*$^{wt-}$ 244 *Lifr*^{tm1c} - 418
Lifr^{tm1d}	5'cas-fwd AAGGCGCATAACGATACCAC	3' loxP-rev ACTGATGGCGAGCTCAGACC Lifr wt-rev2 GGCTGTCCTGGAACTCACTC	62	Type-it	*Lifr*^{tm1c} - 389 *Lifr*^{tm1d} - 172
Pdgfrb-Cre	Pdgfrb-cre fwd TGCCACGACCAAGTGACAGCA Il2-fwd CTAGGCCACAGAATTGAAAGATCT	Pdgfrb-cre rev AGAGACGGAAATCCATCGCTC Il2-rev GTAGGTGGAAATTCTAGCATCATCC	57	Biomix	*Pdgfrb*-cre - 374 *Il2* - 330
Amh-Cre	Amh-cre fwd CACATCAGGCCCAGCTCTAT Il2-fwd CTAGGCCACAGAATTGAAAGATCT	Amh-cre rev GTGTACAGGATCGGCTCTGC Il2-rev GTAGGTGGAAATTCTAGCATCATCC	59	Biomix	*Amh*-cre - 180 *Il2* - 330
Stra8-Cre	Str8-cre fwd GTGCAAGCTGAACAACAGGA Il2-fwd CTAGGCCACAGAATTGAAAGATCT	Stra8-cre rev AGGGACACAGCATTGGAGTC Il2-rev GTAGGTGGAAATTCTAGCATCATCC	59	Biomix	*Stra8*-cre - 260 *Il2* - 330
Td-tomato	td-Tomato-fwd CTGTTCCTGTACGGCATGG Il2-fwd CTAGGCCACAGAATTGAAAGATCT	tdTomato-rev CTGTTCCTGTACGGCATGG Il2-fwd CTAGGCCACAGAATTGAAAGATCT	61	Biomix	*td-Tomato* - 196 *Il2* - 330

2.1.3 Rats

Male Wistar Kyoto rats (Wky/NCrl) at 70-90 days of age were obtained from Charles River (Charles River Laboratories, Margate, UK). The animals were housed in groups of four and maintained in the Biomedical Research Facility Little France (LF2) as described in section 2.1.1. Animals were acclimatised for at least one week prior to entry into experiments.

2.1.4 Treatments

2.1.4.1 Human Chorionic Gonadotrophin

To assess the maximal capacity of Leydig cell testosterone production, animals were treated with human chorionic gonadotrophin (hCG; Chorulon®; Henry Schein, Dumfries, UK). hCG is an agonist for the luteinising hormone/chorionic gonadotrophin receptor (LHGCR), thus stimulates Leydig cell androgen biosynthesis. Animals were given a single 20IU dose of hCG 16hrs prior to sacrifice (see 2.1.5.1). Plasma was harvested as outlined in section 2.1.5.3. Aliquots of stock hCG solution (200IU/mL in saline) were obtained from Central Bioresearch Services within Biomedical Research Facility at Little France and stored at -80^{o}C until use. Aliquots were thawed immediately prior to use and 100µL per animal was injected intraperitoneally.

2.1.4.2 Ethane Dimethanesulphonate

Ethane dimethanesulphonate (EDS) is an alkylating agent and has been widely used to induce Leydig cell-specific ablation and regeneration in the rat testis (Morris *et al.*, 1986). To ablate Leydig cells in preparation for cell transplantation studies, rats were treated with a single dose of EDS (85mg/kg). EDS is not commercially available; the EDS used in experiments herein was a kind gift from Professor Peter O'Shaughnessy (Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow). On the day of injection, EDS was dissolved in sterile dimethyl sulfoxide DMSO (Hybri-Max™; Sigma-Aldrich, Dorset, UK) to give a 200mg/mL stock. Immediately prior to injection a 50mg/mL working solution was prepared by diluting the 200mg/mL stock with DMSO:Water (1:3). Animals were weighed and injected intraperitoneally with 85mg/kg EDS. Animals were monitored closely over the next

48hrs to ensure no adverse reaction to the injection occurred. Control animals were injected with an equivalent volume of vehicle (DMSO:Water).

2.1.4.3 Intratesticular Cell Transplantation

Human adipose-derived perivascular stem cells (hAd-PSC) (see section 2.7.1), were injected into the interstitium of rat testes 4 days after EDS-mediated Leydig cell ablation (see section 2.1.4.2). hAd-PSCs were cultured in Corning® 75cm^2 tissue culture flasks (Sigma-Aldrich, Dorset, UK) for 1 week with or without differentiation-inducing media (see section 2.7.1.1). Cells were harvested from 5 flasks per treatment, pooled, and re-suspended in 250uL Opti-MEM™ reduced serum media (Gibco® Thermo Scientific, Runcorn, UK). Surgeries were performed by Mr William Mungall (Senior Technical Officer - Central Bioresearch Services). In brief, rats were anaesthetized by inhalation of isoflurane. The testes were exposed through a scrotal incision and 50µL cell suspension was injected into each testis, under the tunica albuginea, using a Micro-Fine™+ 0.3mL (30G) syringe (BD Bioscience, Oxford, UK). Testes were returned to the scrotum and the incision closed with Coated VICRYL® (5-0, polyglactin 910) Sutures (Ethicon, Johnson & Johnson Health Care Systems Inc., New Jersey, USA). A single sub-cutaneous injection of analgesic (Buprenorphine; 0.05mg/kg) was administered prior to the animals regaining consciousness. Following surgery, live cell number was quantified in the excess suspension in order to estimate the number of cells transplanted. On average, approximately 1.8 (±0.62) million cells were injected into each testis.

Following sacrifice and tissue collection (see section 2.1.5), genomic DNA (gDNA) was isolated from testes and interrogated for the presence/absence of human-specific genomic DNA by PCR as described by (Caspi *et al.*, 2007). Briefly, gDNA from four separate samples per testis was isolated using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Runcorn, UK) following the manufacturer's instructions. Multiplex PCR amplification of α-satellite region of human chromosome 17 (*HUMSAT17A*) and rat beta actin (*Actb*) was performed using the Type-it Mutation Detect PCR Kit (QIAGEN Ltd., Crawley, UK) according to the manufacturer's instructions. The primer sequences were *HUMSAT17A*-FWD GGGATAATTTCAGCTGACTAAACAG, *HUMSAT17A*-REV GTGTTTCATAGCTGCTCTTTCCA and *Actb*-FWD TGTGTTCTTGCCCTCTTTGC, *Actb*-REV CAGGAAGGAAGGCTGGAAGA.

PCR products were analysed using the QIAxcel capillary electrophoresis system (QIAGEN Ltd., Crawley, UK). A reaction containing no template (NTC) was included to ensure reagents were free from contaminating nucleic acids. Genomic DNA extracted from pre-grafted cells was also included as a positive control.

2.1.5 Tissue Collection

2.1.5.1 Sacrifice

Animals were sacrificed by methods conforming to Schedule 1 of the Animals (Scientific Procedures) Act, 1986. For adult animals, this was achieved by exposure to an increasing concentration of carbon dioxide, followed by confirmation of permanent cessation of the circulation by palpitation. For embryonic ages, pregnant dams were dispatched as described above, and the fetuses rapidly removed and decapitated.

2.1.5.2 Dissection

Animals were weighed prior to blood collection (detailed in 2.1.5.3) using a digital balance (Mettler Toledo, London, UK). Testes, epidiymides and seminal vesicles (including coagulating glands) were removed and trimmed of surrounding adipose tissue. The weights of the testes and seminal vesicle (intact including seminal fluid) were recorded. Ovaries and uteri were collected from female animals as well as a panel of extra gonadal tissues (from both sexes) including pituitary, adrenal, pancreas, liver, kidney and spleen.

2.1.5.3 Tissue Processing and Storage

Blood was collected immediately after sacrifice *via* cardiac puncture using a 23G needle fitted to a 1 mL syringe (BD Bioscience, Oxford, UK) pre coated with heparin (LEO Laboratories Ltd., Hurley, UK). Plasma was harvested by centrifugation at 17136g for 10mins and stored at either -20^{o}C (short-term) or -80^{o}C (long term). Organs were either snap frozen on dry ice and stored at -80^{o}C for molecular analyses, or fixed in Modified Bouin's Solution (Clin-Tech Ltd, Guildford, UK) for subsequent histological analyses. In the case of the testes; the left testis was cut in half along the axial plane, roughly through the vascular plexus overlying the rete testis, and both halves were snap frozen immediately. The right testis was submerged in Bouin's solution for 6hrs at room temperature. After an initial 4hrs, the testis was cut in half

along the axial plane to ensure fixative penetration to the centre of the organ during the final 2hrs of fixation. Fixed samples were then stored in 70% ethanol (EtOH; VWR, Lutterworth, UK) until processing and embedding. Frozen tissues and Bouin's-fixed samples from naturally aged mice (18 months old) were obtained from the MRC Harwell Ageing Screen, through collaboration with Dr. Paul Potter (MRC Harwell, Oxfordshire, UK).

For tissues expressing fluorescent proteins, organs were dissected into cold phosphate buffered saline (PBS, see section 2.9.5; Sigma-Aldrich, Dorset, UK) and imaged using a Leica M2FIII fluorescence stereomicroscope (Leica Microsystems, Milton Keynes, UK) prior to fixation as described above.

Where integrity of the blood testis barrier was to be assessed, a biotin tracer (EZ-link sulfo-NHS-LC-biotin; Thermo Scientific, Runcorn, UK) was injected into the testis. Briefly, 10-15µL of freshly prepared biotin solution (10mg/mL in 0.01M $MgCl_2$ in PBS) was injected under the tunica albuginea of freshly collected testes. Contralateral testes were injected with vehicle only (0.01M $MgCl_2$ in PBS). Testes were incubated on ice for 30mins and then fixed and processed as described above. Localisation of the biotin tracer was visualised in testis sections by incubation with Streptavidin Alexa Fluor™ 546 conjugate (S11225, Thermo Scientific, Runcorn, UK), diluted 1:200 in PBS, for 1hr at room temperature. Tissue sections were imaged using an LSM 780 confocal microscope (Carl Zeiss, UK).

2.2 Histological Analyses

2.2.1 Tissue Sectioning

Fixed tissues were subject to an automated tissue processing procedure using the Leica P1050 processing system (Leica Biosystems, Milton Keynes, UK). During this process, samples were dehydrated through an ascending series of ethanol, cleared through xylene (VWR, Lutterworth, UK) and impregnated with Shandon™ Paraffin (Thermo Scientific, Runcorn, UK). Processed tissues were then embedded in molten paraffin. Processing and embedding was carried out by Mr Garry Menzies – Technical Officer within the Shared University Research Facilities core (SuRF), Edinburgh.

Once cooled, the paraffin blocks were stored at room temperature in a tissue archive maintained by the SuRF histology facility at Little France.

Paraffin embedded tissue sections were cut at a thickness of 5μm using a Leica RM2125RTS rotary microtome (Leica Biosystems, Milton Keynes, UK). Sections were mounted onto positively charged X-tra® microscope slides (Leica Biosystems, Milton Keynes, UK) and dehydrated in an oven at 50ºC overnight (~16hrs). Slides were then allowed to cool before being stored at room temperature for use in downstream staining applications detailed in sections 2.2.2 and 2.2.3.

2.2.2 Haematoxylin and Eosin Staining

Gross histological architecture was examined in prepared tissue slides using the nuclear and cytoplasmic stains haematoxylin and eosin (H&E) respectively. The staining procedure is detailed in Figure 2.3.

2.2.2.1 Dewaxing and Rehydration

In preparation for use in histological and immunohistochemical staining experiments, tissue sections were dewaxed and rehydrated using standard procedures outlined in Figure 2.3. Briefly, slides were immersed in xylene to remove paraffin wax, passed through a decreasing series of ethanol to displace the xylene and washed in tap water to fully rehydrate the tissue.

2.2.2.2 Staining

Following dewaxing and rehydration (detailed in 2.2.2.1), slides were immersed in Harris haematoxylin (Leica Biosystems, Milton Keynes, UK) to stain nuclei. Slides were then immersed in 1% acid-alcohol (Leica Biosystems, Milton Keynes) to remove background cytoplasmic staining. To increase contrast, the nuclear staining was then developed from purple to blue by immersing the slides in Scott's tap water substitute (Cell Path, Powys, UK). Slides were then immersed in Eosin Y solution (Leica Biosystems, Milton Keynes, UK) to stain basophilic structures, such as cell cytoplasm, with various pink/orange hues. Following staining, slides were dehydrated and cleared as outlined in Figure 2.3. Briefly, the tissue sections were passed through an ascending series of ethanol and then cleared of alcohol by immersion in xylene. Finally, slides

were mounted with glass cover slips (Leica Biosystems, Milton Keynes) using the non-aqueous mounting medium, Pertex (Cell Path, Powys, UK).

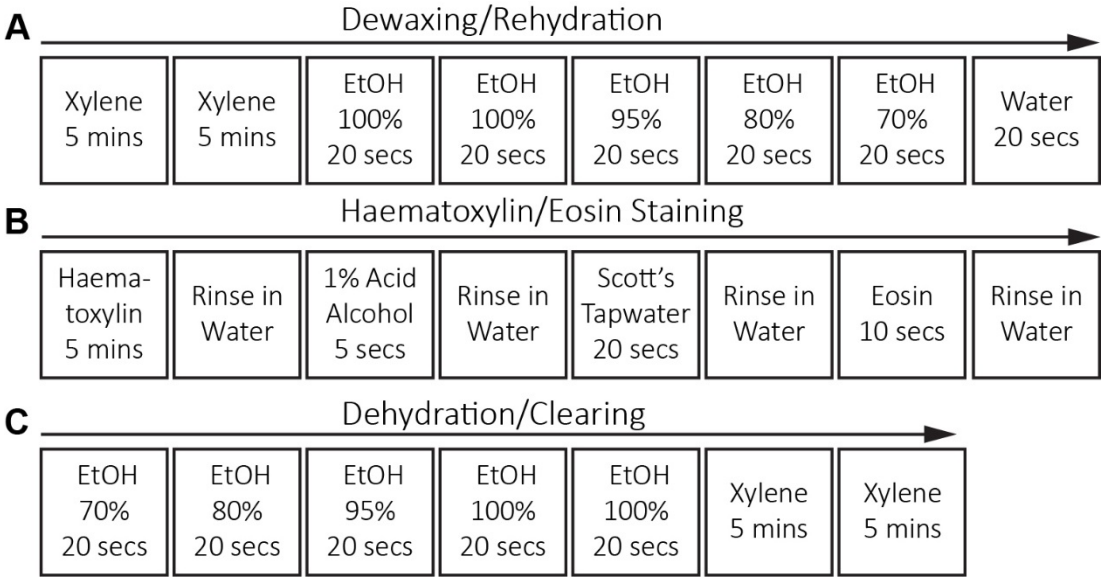


Figure 2.3 Haematoxylin & Eosin Staining Procedure. (A) Sections are dewaxed in xylene and rehydrated through a series of graded ethanol to water. (B) Haematoxylin and Eosin stain nuclei and cytoplasm respectively. (C) Sections are dehydrated through a series of graded ethanol, and cleared in xylene prior to mounting.

2.2.3 Immunohistochemistry

In order to visualise the localisation of specific proteins on tissue sections, immunohistochemistry (IHC) experiments were performed. For simplex detections, chromogenic IHC was generally performed, whereas fluorescent IHC was used for multiplex detections. Details of the antibodies used are shown in Table 2-2. In the first instance, an initial optimisation step was performed in order to determine the appropriate conditions for each antibody (i.e. antibody dilution, antigen retrieval method and secondary detection reagent).

2.2.3.1 Antigen Retrieval

One way in which tissue fixation preserves tissue structure is by cross-linking proteins. However, this can lead to masking of specific epitopes recognised by primary antibodies. As such, an antigen retrieval step may be required for optimal antigen detection (Pileri *et al.*, 1997, Shi *et al.*, 2001). For IHC experiments described in this thesis, a heat-induced epitope retrieval (HIER) was used. Following dewaxing and rehydration (detailed in 2.2.2.1), slides were boiled in a pressure cooker (Instant Pot,

Burnaby, CA) in either pH 6.0 citrate buffer (section 2.9.2.2) or pH 9.0 Tris-EDTA buffer (see section 2.9.3) for 5mins. Retrieval methods for the antibodies used herein are noted in Table 2-2. *Table on following Page*

Table 2-2. Details of the Antibodies used for Immunohistochemistry

Target	Antigen Retrieval	Primary Antibody			Secondary Reagent			Detection Method
		Source	Catalogue No.	Dilution	Source	Catalogue No.	Dilution	
HSD3b	Citrate pH 6	Santa Cruz Biotechnology *Heidelberg, Germany*	sc30820	1:2000	Vector Laboratories *Burlingame, CA, USA*	MP-7405	n/a	ImmPRESS™ HRP DAB
HSD3b	Citrate pH 6	Santa Cruz Biotechnology *Heidelberg, Germany*	sc30820	1:2000	Santa Cruz Biotechnology *Heidelberg, Germany*	sc-2961	1:200	ChαG HRP Tyramide
SOX9	Tris-EDTA pH 9	Merk Millipore *Billerica, MA, USA*	AB5535	1:4000	Vector Laboratories *Burlingame, CA, USA*	MP-7451	n/a	ImmPRESS™ HRP DAB
SOX9	Tris-EDTA pH 9	Merk Millipore *Billerica, MA, USA*	AB5535	1:4000	Vector Laboratories *Burlingame, CA, USA*	PI-1000	1:200	GαR HRP Tyramide
RFP	n/a	Evrogen *Moscow, Russia*	AB233	1:800	Vector Laboratories *Burlingame, CA, USA*	BA-1000	1:500	Streptavidin HRP DAB
RFP	Citrate pH 6	Clontech Laboratories Inc. *Montain View, CA, USA*	632496	1:2000	Vector Laboratories *Burlingame, CA, USA*	PI-1000	1:200	GαR HRP Tyramide
YFP	Citrate pH 6	Abcam *Cambridge, UK*	ab6556	1:4000	Vector Laboratories *Burlingame, CA, USA*	PI-1000	1:200	GαR HRP Tyramide
CASP3	n/a	Cell Signalling Technology *Leiden, Netherlands*	9661S	1:300	Anti-rabbit Poly-HRP-IgG *Leica Biosystems, UK*	DS 9800	n/a	Poly-HRP DAB
COUP-TFII	Citrate pH 6	Persus Proteomics Inc. *Tokyo, Japan*	PP-H7147-00	1:1000	Agilent Technologies (DAKO) *Cheadle, UK*	P0447	1:500	GαM HRP Tyramide
DDX4	Citrate pH 6	Abcam *Cambridge, UK*	ab13840	1:1000	Vector Laboratories *Burlingame, CA, USA*	PI-1000	1:200	GαR HRP Tyramide
PDGFRb	Citrate pH 6	Abcam *Cambridge, UK*	ab32570	1:1500	Vector Laboratories *Burlingame, CA, USA*	PI-1000	1:200	GαR HRP Tyramide
HLA	n/a	Abcam *Cambridge, UK*	ab70328	1:80	Thermo Scientific *Runcorn, UK*	A-11030	1:200	GαM Alexa Flour 546
HuNu	n/a	Merk Millipore *Billerica, MA, USA*	MAB1281	1:320	Thermo Scientific *Runcorn, UK*	A-11030	1:200	GαM Alexa Flour 546

ChαG = Chicken anti-Goat; GαR = Goat anti-Rabbit; GαM = Goat anti-Mouse; HRP = Horseradish Peroxidase; DAB = 3, 3'-diaminobenzidine.

2.2.3.2 Chromogenic Detection

Prior to the addition of primary antibodies, endogenous peroxidase activity was quenched by immersing slides in 0.3% (w/vol) hydrogen peroxide (Fisher Scientific, Loughborough, UK) in TBS (see section 2.9.4.2) for 30mins at room temperature. All subsequent incubations were carried out in a humidity chamber. Unless otherwise stated, slides were subjected to 3x5min washes in TBS between each incubation step. Binding of antibodies to non-specific epitopes was reduced by incubating sections with a normal serum blocking solution (see section 2.9.6.1), of the same species in which the secondary antibody was raised, for 30mins at room temperature. Tissue sections were then incubated with primary antibody, diluted in blocking solution, overnight (~16hrs) at 4^{o}C. Next, sections were incubated with either a biotin-conjugated secondary antibody specific to the species of the primary antibody (see Table 2-2), diluted 1:500 in blocking solution, followed by a streptavidin-horseradish peroxidase conjugate (SA-5004; Vector Labs, Burlingame, CA, USA) diluted 1:1000 in TBS; or with the appropriate ImmPRESS™ peroxidase polymer detection reagent (Vector Labs, Burlingame, CA, USA). Localisation of primary antibody was visualised by the addition of the chromogenic peroxidase substrate 3, 3'-diaminobenzidine (DAB; SK-4105, Vector Labs, Peterborough, UK) according to the manufacturer's instructions. Colour development was timed on control slides and the remaining slides were developed concurrently based on this time. Chromogenic reactions were stopped by immersing slides in tap water. Tissue sections were then counterstained with Harris haematoxylin, dehydrated and mounted with glass coverslips as described in section 2.2.2. Control sections from which the primary antibody had been omitted were included in each experiment. Depending on availability, further controls were also run in each experiment which included pre-incubation of the primary antibody with the peptide it was raised against and/or a tissue section known to be negative for the antigen of interest.

2.2.3.2.1 Automated Chromogenic Detection

In certain instances (i.e. for the detection of CASP3), chromogenic immunohistochemistry was performed using the Bond™ Polymer Refine Detection system (Leica Biosystems, Milton Keynes, UK) in conjunction with the BOND-MAX

automated staining robot (Leica Biosystems, Milton Keynes, UK) according to the manufacturer's instructions.

2.2.3.3 Fluorescent Detection

For Fluorescent IHC, tissue sections were treated as described for chromogenic IHC (see section 2.2.3.2) until the addition of the secondary antibody. Following overnight incubation at 4^{o}C with the primary antibody, sections were incubated with the appropriate peroxidase-conjugated secondary antibody (Table 2-2), diluted 1:200 in blocking solution, for 30mins at room temperature. Sections were then incubated for 10mins at room temperature with the fluorogenic Tyramide Signal Amplification System (TSA; PerkinElmer, UK), diluted 1:50 in the kit substrate as per the manufacturer's instructions. All subsequent steps were carried out with limited light exposure to prevent degradation of fluorescent dyes. Sections were next incubated with SYTOX® Green nucleic acid stain (Molecular Probes, Life Technologies, Paisley UK), diluted 1:10,000 in TBS, for 10mins. Slides were then mounted with glass cover slips (Leica Biosystems, Milton Keynes) using Lab Vision™ PermaFluor™ aqueous mounting medium (Thermo Scientific, Runcorn, UK). Controls as described in section 2.2.3.2 were included. Stained slides were stored in the dark at 4^{o}C until imaging (section 2.2.5)

2.2.3.4 Multiplex Antigen Detection

In order to visualise the localisation of multiple proteins on the same tissue section, multiplex fluorescent IHC experiments were performed. Following detection of the primary antibody as described in section 2.2.3.3, sections were subjected to further HEIR, peroxidase quenching and non-specific epitope blocking steps, as described in sections 2.2.3.1 and 2.2.3.2, before overnight incubation at 4^{o}C with a second primary antibody. Primary antibody detection and nuclear counterstaining were carried out as described in section 2.2.3.3. Controls as described in section 2.2.3.2, were included. Stained slides were stored in the dark at 4^{o}C until imaging (see section 2.2.5).

2.2.4 Fluorescent Immunocytochemistry

In order to assess the expression of specific proteins in cultured cells, fluorescent immunocytochemistry experiments were performed. Cells were grown and stained using the Nunc™ Lab-Tek™ II Chamber Slide™ System (Thermo Scientific, Runcorn,

UK). Culture media was removed and the cells were washed twice with PBS. Cells were fixed by incubation with 4% paraformaldehyde (PFA; Thermo Scientific, Runcorn, UK) for 10mins at 4ºC. After washing twice with PBS, cells were permeablised by incubation with PBS containing 0.1% (vol/vol) Triton™ X-100 (Sigma-Aldrich, Dorset, UK) for 10mins at 4ºC. Binding of antibodies to non-specific epitopes was reduced by incubating sections with a normal serum blocking solution (see section 2.9.6.1), of the same species in which the secondary antibody was raised. Cells were then incubated with primary antibody, diluted in blocking solution, overnight (~16hrs) at 4ºC. After washing twice with PBS, cells were incubated with the appropriate fluorophore-conjugated secondary antibody (see Table 2-2) for 1hr at room temperature. Cells were then washed twice with PBS and incubated with DAPI nucleic acid stain (Sigma-Aldrich, Dorset, UK). Slides were then mounted with glass cover slips (Leica Biosystems, Milton Keynes) using Lab Vision™ PermaFluor™ aqueous mounting medium (Thermo Scientific, Runcorn, UK). Controls as described in section 2.2.3.2 were included. Stained slides were stored in the dark at 4ºC until imaging (see section 2.2.5).

2.2.5 Microscopy and Image Capture

Images were captured using either an Axio Scan Z.1 slide scanner (Carl Zeiss Ltd, Welwyn Garden City, UK) or an LSM 780 confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) with Zen imaging software (Carl Zeiss Ltd, Welwyn Garden City, UK).

2.2.6 Stereological Analysis

Leydig and Sertoli cells were identified in tissue sections by chromogenic immunostaining (see section 2.2.3.2) for HSD3b and SOX9 respectively. Quantification of cells was conducted using the stereology plug-in for Image-Pro plus 7.0 software (Media Cybernetics, UK) and a Zeiss Axio Imager A1 microscope (Carl Zeiss, UK) fitted with a Qimaging QICAM Fast 1394 digital camera (Qimaging, Canada) and a Prior ProScan automated stage (Prior Scientific Instrument Ltd, UK). For each sample, a tiled image encompassing the entire testis cross-section was generated and highlighted as the area of interest (AOI). 200-300 random fields were generated within the AOI for interrogation at 630X magnification. The software then

drove the stage between random fields, a counting grid (18 x 24) was overlaid onto the tissue and the 'count' function was used to score points falling over nuclei of immuno-positive cells. Only fields containing ≥50% tissue area were scored. Typically, 100 or 30 fields per section were analysed for Leydig and Sertoli cells respectively. This ensured the standard error (SE) for counting remained <10%. The SE was calculated using the formula:

$$SE = \sqrt{(Ppi(100-Ppi)/Pt)}$$

$$SE\ (\%) = (SE/Ppi) \times 100$$

(*Where Ppi = the number of points falling over nuclei as a percentage of the total number of points counted and Pt = the total number of points counted*).

These data, which reflect the relative volume occupied by the object of interest (e.g. Leydig or Sertoli cell nuclei), were then converted into absolute volume using testis weight:

$$\text{Absolute volume} = (Ppi \times \text{Testis Weight [mg]})/100$$

The 'nucleator' function in the stereology plugin, was then utilised to obtain the mean nuclear volume (MNV) of each cell type in the same sections used for point counting. Briefly, random fields within the AOI were generated as described above. At 630X magnification, the centre of the nucleus was selected and three random lines intersecting the nuclear membrane and the centre of the nucleus were generated. The point(s) at which each line intersected the nuclear boundary were selected, generating 6 random radii measurements which were averaged and used to extrapolate the nuclear volume. Typically, measurements were obtained from approximately 150 nuclei per sample. The absolute volume and MNV were then used to calculate the number of each cell type per testis using the following formula:

$$\text{Number of Cells per Testis} = (\text{Absolute Volume [mg]}/\text{MNV [}\mu m^3\text{]}) \times 1000$$

Seminiferous tubule and lumen diameters were also measured using the stereology 'nucleator' function as described for the MNV. However, these measurements were performed at 100X magnification, across two sections from the same sample. Only circular tubules were scored and care was taken to avoid scoring tubules in sections

with obvious fixation-induced shrinkage artefact. Typically, 100 tubules per sample were scored.

I am very grateful to Professor Nina Atanassova (Institute of Experimental Morphology, Pathology and Anthropology with Museum, Sofia, Bulgaria) who carried out the quantification of Sertoli and germ cell absolute nuclear volume in Sertoli cell LIFR-KO testes (Chapter 4).

2.3 Evaluation of the Epididymal Sperm Reserve

Epididymal sperm reserves were quantified from frozen epididymides. Epididymides were thawed on ice and the cauda epididymis was separated, taking care to ensure consistency in sampling through the corpus epididymis and vas deferens. The cauda epididymis was then homogenised on ice in 500µL of homogenisation buffer (0.9% NaCl, 0.5% Triton X-100) using an Eppendorf® micro-pestle (Sigma-Aldrich, Dorset, UK). If necessary, the sample was further diluted in homogenisation buffer to ensure accurate counting. Homogenisation-resistant elongate spermatids were quantified using a Neubauer haemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany). Briefly, 15-25µL of homogenate was loaded into the two counting chambers of the haemocytometer and allowed to settle for 2-3mins before counting. To reduce error, at least 5 quadrants from the counting grid in each chamber were counted and the number averaged to give the number of elongate spermatids in 0.004µL. This value was then multiplied by $2.5x10^4$ to arrive at the number of spermatids per mL. Finally, the dilution factor applied to the original homogenate was corrected for. I gratefully acknowledge the assistance of Mr Nathan Jeffery who carried out the sperm counts.

2.4 Hormone Analysis

2.4.1 Mouse samples

The quantification of luteinising hormone in mouse plasma was carried out using the Milliplex MAP Pituitary Magnetic Bead Panel Kit (PPTMAG-86K; Merk Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Samples were read on a Bio-Plex® 200 suspension array system with Bio-Plex Manager™ software (Bio-

Rad Laboratories Ltd., Watford, UK). I gratefully acknowledge the assistance of Dr Laura Milne who carried out the assays.

The quantification of testosterone in mouse plasma was carried out using an isotope-dilution liquid chromatography-mass spectrometry (LC-MS/MS) method as described by Søeborg *et al.* (2017). LC-MS/MS was performed by collaborators in the Department of Growth and Reproduction, Rigshospitalet, Copenhagen University Hospital. I am particularly grateful to Dr Hanne Frederiksen and Dr Anne Jørgensen for their assistance.

2.4.2 Rat samples

The quantification of luteinising hormone in rat plasma was carried out using an in-house ELISA as previously described (Tyndall *et al.*, 2012). Testosterone was quantified in rat plasma using an in house competitive ELISA. Briefly, 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with 100µL of donkey anti rabbit IgG (Jackson ImmunoResearch Inc, West Grove, USA) per well at a dilution of 1:500 in ELISA coating buffer (100mM $NaHCO_3$, pH 9.6). After overnight incubation at 4ºC, plates were washed twice with wash buffer (0.05M Tris-HCl, 0.05% Tween 20, pH 7.4) and incubated with 250µL of blocking buffer (PBS pH 7.4 containing 0.5 % BSA) for 1hr at room temperature. Plates were then washed twice with wash buffer prior to the addition of standards, samples and controls (20µL per well), followed by 80µL of testosterone-HRP conjugate (Astra Biotech GmbH, Berlin, Germany) at 1:20,000 in assay buffer (PBS pH 7.4 containing 0.1 % BSA and 250 ng/mL Cortisol), followed by 50µL of rabbit anti-testosterone-19 antibody (AMS Biotechnology, USA) at 1:200,000 in assay buffer. Plates were incubated at room temperature for 2 hr on an IKA®, Schuttler MTS4 plate shaker (IKA Labortechnik, Staufen, Germany), then washed 5 times with assay wash buffer. Next, 120µL of substrate solution (3,3,5,5-Tetramethylbenzidine, Millipore Corporation, Temecula, CA, USA) was added to each well and plates were incubated at room temperature, without shaking, in the dark, for 20mins. The reaction was stopped by the addition of 80µL stop solution (2N H_2SO_4; Sigma-Aldrich, Dorset, UK). Finally, plates were read on a plate reader at 450nm. Assays were carried out by Dr Forbes Howie and Miss Kirsten Wilson in the SuRF assay lab.

2.5 mRNA Expression Analysis

2.5.1 RNA Extraction

Total RNA was extracted from frozen tissues or from cell cultures using the RNeasy® Mini kit (QIAGEN Ltd., Crawley, UK) as per the manufacturer's instructions including an on column DNase (QIAGEN Ltd., Crawley, UK) treatment step. In the case of the testis, an external control *Luciferase* RNA (5ng/testis; Promega, Southampton, UK) was added to each sample prior to extraction. RNA purity and concentration were determined using a NanoDrop 1000 v3.8.1 spectrophotometer (Thermo Scientific, Runcorn, UK) with a 260:280 absorbance ratio of ~2.0 indicative suitably pure RNA. Resulting RNA was stored in RNase free tubes at -80ºC.

2.5.2 cDNA Synthesis

Random hexamer-primed cDNA was reverse transcribed from up to 2µg total RNA (see section 2.5.1) using the SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Paisley, UK) as per the manufacturer's instructions. Incubations were carried out using a Tprofessional thermocycler (Biometra GmbH, Göttingen, Germany). A reverse transcriptase negative (-RT) control was included to ensure reagents were free of nucleic acid contamination. The resulting cDNA was stored at -20ºC.

2.5.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

For non-quantitative analysis of mRNA expression, RT-PCR was performed. cDNA (see section 2.5.2) was amplified using BioMix™ PCR reaction buffer (Bioline Reagents Ltd, London, UK) according to the manufacturer's instructions. Target specific primers were designed using Primer 3 online tool (http://primer3.ut.ee/), and ordered from Eurofins MWG Operon (Eurofins Genomics, Ebersberg, Germany). Optimal primer annealing temperature was determined using the gradient function on the thermocycler. PCR products were analysed using the QIAxcel capillary electrophoresis system (QIAGEN Ltd., Crawley, UK). A reaction containing no template (NTC) was included to ensure reagents were free from contaminating nucleic acids.

2.5.4 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

For quantitative analysis of mRNA expression, qRT-PCR was performed on cDNA (see section 2.5.2) using the Universal ProbeLibrary (UPL; Roche Diagnostics Ltd., Burgess Hill, UK). Briefly, UPL probes are short (8-9 nucleotide) hydrolysis probes harbouring a 5' fluorescent reporter and a 3' quencher which acts to prevent reporter emission. During PCR cycling, the UPL probe binds the target amplicon and the 5'-3' nuclease activity of DNA polymerase liberates the reporter from the quencher; resulting in fluorescent emission which is recorded by a suitable detector. During PCR cycling, the fluorescent signal increases proportionally with the amount of amplified product. As PCR cycling proceeds, the fluorescence is recorded and plotted against the cycle number (Figure 2.4). During the exponential amplification phase of the PCR reaction, as the amplicon accumulates, the fluorescent signal becomes readily detectable above background levels. It is in this phase, when reagents are not limiting, that the threshold cycle (Ct) is set (Figure 2.4). The Ct value is inversely related to the amount of template at the start of the reaction thus reflects the mRNA expression level in the original sample.

The online Universal ProbeLibrary Assay Design Centre was used to select target-specific primers and corresponding UPL probes. Where possible, assays were designed across an intron to avoid the amplification of potential genomic DNA contaminants. qRT-PCR assays were carried out in 384-well format on the ABI Prism 7900HT Real-Time PCR System with SDS 2.4 software (Applied Biosystems, Life Technologies, Paisley, UK), using the Applied Biosystems™ TaqMan™ Universal PCR Master Mix (Applied Biosystems, Life Technologies, Paisley, UK) according to the manufacturer's instructions. Samples were assayed in triplicate. Reverse transcriptase negative (section 2.5.2) and no template controls were included to ensure reagents were free of contamination. Details of the assays used in these studies, including primer sequences, UPL probe number and amplification efficiency, are listed in Table 2-3. The external *Luciferase* control was assayed using gcacatatcgaggtgaacatcac and gccaaccgaacggacattt forward and reverse primers respectively and, a 5'NED labelled probe (tacgcggaatacttc).

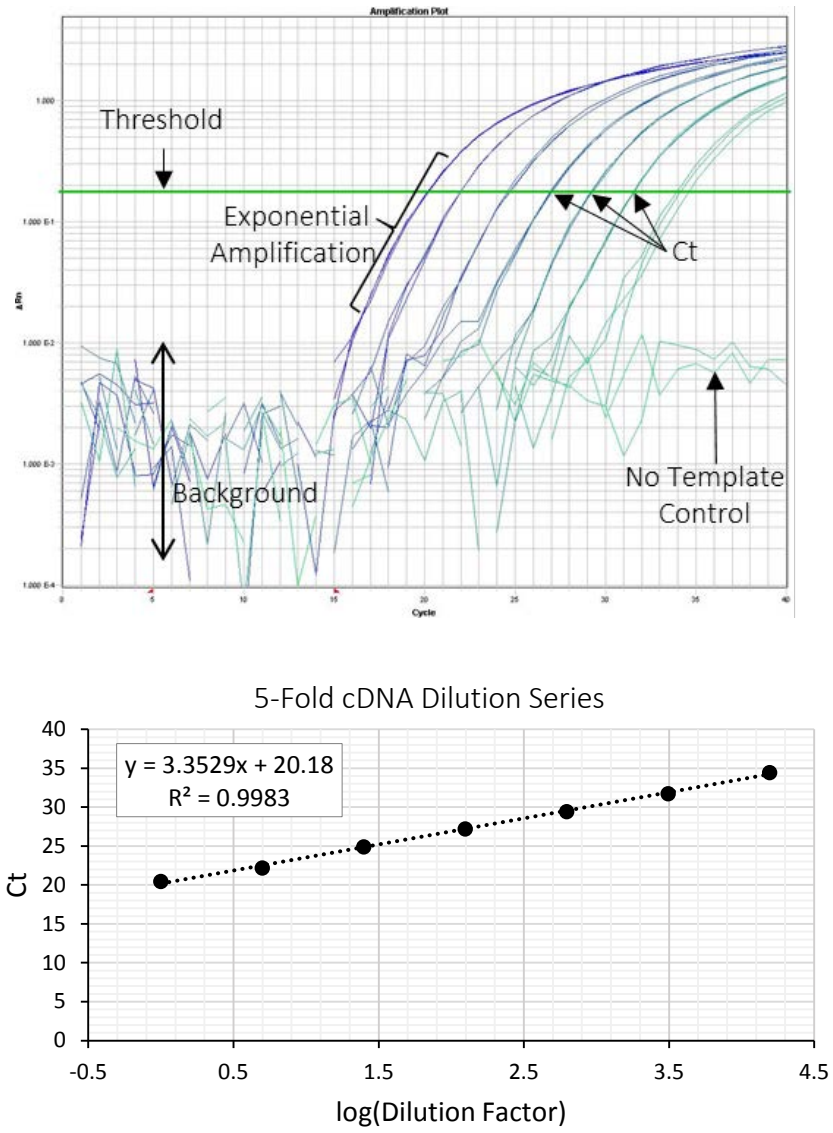


Figure 2.4 qRT-PCR Assay Validation. Top panel shows representative amplification plot of a 5-fold dilution series of template cDNA. Bottom panel shows the threshold cycle (Ct) plotted against log-template concentration. The slope of the line is used to calculate assay efficiency.

Table 2-3. Details of Primers and Probes used for qRT-PCR Assays

Gene	Forward Primer	Reverse Primer	UPL Probe	Efficiency (%)
Lhcgr	gatgcacagtggcaccttc	cctgcaatttggtggaagag	107	96
Star	aaactcacttggctgctcagta	tgcgataggacctggttgat	83	99
Cyp11a1	aagtatggccccatttacagg	tggggtccacgatgtaaact	104	98
Hsd3b1	gaactgcaggaggtcagagc	gcactgggcatccagaat	12	98
Hsd3b6	accatccttccacagttctagc	acagtgaccctggagatggt	95	101
Cyp17a1	catcccacacaaggctaaca	cagtgcccagagattgatga	67	94
Hsd17b3	aatatgtcacgatcggagctg	gaagggatccggttcagaat	5	95
Ocdn	gcggaaagagttgacagtcc	atctcctgggccacttcag	25	99
Cldn3	tgggagctgggttgtacg	caggagcaacacagcaagg	26	99
Cldn11	tggagtggccaagtacagg	gacaatggcgcagagagc	20	93
Stra8	ttgacgtggcaagtttcct	agttgcaggtggcaaacata	107	102
Spo11	ggctcctggacgacaactt	cagatctggaacgcccttt	18	99
Tpn1	agccgcaagctaaagactca	cggtaattgcgacttgcat	91	96
Gdnf	gctcaaaattgtgacaacctca	cagagggtctggaacgacat	107	104
Cyp26b1	aacatggcaaggagatgacc	ttgcatgatcaaggatgtgc	17	99
Kitl	gctgctggtgcaatatgct	gataaatgcaagtgataatccaagttt	50	97
STAR	ggcatccttagcaaccaaga	tctgggaccactttactcatca	18	104
CYP11A1	gatgacctgttccgctttg	cctcggggttcactacttcc	89	102
CYP17A1	ctatgctcatcccccacaag	ccttgtccacagcaaactca	67	108*
HSD17B3	aacttgcaggcttagaaattgg	ggtgcgttcaggaaatgg	7	108*

** These assays were not used for quantitative analyses*

2.5.4.1 Assay Validation

As described in section 2.5.4, quantification of mRNA expression by qRT-PCR relies on the relationship between initial target abundance and the Ct value obtained during exponential amplification. As such, it is absolutely necessary that the PCR reaction is suitably efficient in order to obtain reliable results. Assays with an efficiency between 90 and 105% were considered fit for purpose. In order to establish the efficiency of each assay, a serial dilution of cDNA was prepared and amplified with appropriate primers and probe. Following amplification, the Ct value was plotted against the log dilution factor (Figure 2.4). The equation of the linear regression line was then used to calculate the efficiency of the reaction using the following formulas:

$$\text{Exponential amplification (E)} = 10^{-1/\text{slope}}$$

$$\text{Reaction Efficiency (\%)} = (E-1) \times 100$$

In an ideal situation, there would be perfect doubling of the amplicon during each cycle (i.e. a reaction efficiency of 100%). In the example shown in Figure 2.4, the efficiency was calculated to be 98.7%:

$$E = 10^{-1/3.3529} = 1.987235$$

$$\text{Reaction Efficiency} = (1.987235-1) \times 100 = 98.7\%$$

2.5.4.2 Analysis

Relative quantification of mRNA expression was carried out using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001, Schmittgen and Livak, 2008). Briefly, the Ct of the external control (*Luciferase*) was subtracted from the Ct of the target to give the ΔCt. The triplicates for each sample were then averaged, and corrected to a reference (control ΔCt) to give the ΔΔCt. This was achieved by subtracting the reference ΔCt from the ΔCt of each sample. The fold-change between control and experimental groups was then calculated using the formula $2^{-\Delta\Delta Ct}$.

2.6 Western Blotting

2.6.1 Protein Extraction

Protein was extracted from frozen tissues collected and stored as described in section 2.1.5. A piece of tissue, approximately 30mg, was used for extractions. Samples were

homogenised in 500µL RIPA buffer (15mM HEPES-NaOH [pH 7.5], 0.5mM NaCl, 1mM sodium orthovanadate, 10mM EDTA; 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulphate) supplemented with cOmplete™ MINI protease inhibitors and PhosSTOP™ protease inhibitors (Roche Diagnostic Ltd., Burgess Hill,UK) using a Retsch tissue lyser (Qiagen QIAGEN Ltd., Crawley, UK). The homogenate was incubated on ice for 1hr before centrifugation at 2500rpm for 10mins at 4^{o}C. The resulting supernatant was stored in aliquots at -80^{o}C. Protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories Ltd., Watford, UK) following the manufacturer's instructions.

2.6.2 Protein Electrophoresis and Transfer

Protein samples in NuPage LDS sample buffer (Life Technologies, Paisley UK) containing NuPage reducing agent (Life Technologies, Paisley UK) were heated to 70^{o}C for 5mins and loaded onto Novex® Tris-Glycine or Tris-Acetate polyacrylamide gels (Life Technologies, Paisley UK). Samples were electrophoresed under denaturing conditions in NuPAGE MOPS SDS or NuPage Tris-Acetate SDS running buffer (Life Technologies, Paisley UK) at 200V for approximately 45mins using the XCell SureLock™ Mini-Cell Electrophoresis System (Thermo Scientific, Runcorn, UK). Proteins were then transferred in NuPage transfer buffer (Life Technologies, Paisley UK) onto Immobilon-FL PVDF membranes (Merk Millipore, Billerica, MA, USA) at 400V for 90mins. Appropriate care was taken to ensure buffers remained as cool as possible during electrophoresis and transfer steps.

2.6.3 Protein Detection

Membranes were washed in PBS. Non-specific epitopes were blocked by incubating membranes with PBS containing 5% (w/v) semi-skimmed milk powder (Premier Foods Ltd., St. Albans, UK) for 1hr at room temp. Membranes were then incubated with primary antibodies diluted in blocking buffer at 4^{o}C overnight.

Membranes were then subjected to 3x5min washes with PBS containing 0.1% (v/v) Tween® 20 (PBST; Sigma-Aldrich Co. Ltd., Dorset, UK) before incubation with the appropriate IRDye® secondary antibodies (LI-COR Biotechnology Ltd, Cambridge, UK), diluted in blocking buffer, for 1hr at room temp. Finally, membranes were washed in PBST and stored in PBS in the dark at 4^{o}C until being read on a LI-COR

Odessey Infra-red scanner (LI-COR Biotechnology Ltd, Cambridge, UK). Details of antibodies used for Western blotting are listed in Table 2-4. *Table on following page.*

Table 2-4. Details of the Antibodies used for Western Blotting

Target	Primary Antibody			Secondary Antibody			
	Source	Cat. No.	Dilution	Label	Source	Cat. No.	Dilution
CISD2	Proteintech *Manchester, UK*	13318-1-AP	1:500	Donkey anti-Rabbit IRDye® 800CW	LI-COR Biotechnology *Cambridge, UK*	925-32213	1:10000
LIFR	Santa Cruz Biotechnology *Heidelberg, Germany*	sc-659	1:400	Goat anti-Rabbit IRDye® 800CW	LI-COR Biotechnology *Cambridge, UK*	925-32211	1:10000
GAPDH	Abcam *Cambridge, UK*	ab9483	1:4000	Donkey anti-Goat IRDye® 680RD	LI-COR Biotechnology *Cambridge, UK*	925-68074	1:10000
TUBa	Abcam *Cambridge, UK*	ab6160	1:5000	Goat anti-Rat IRDye® 680RD	LI-COR Biotechnology *Cambridge, UK*	925-68076	1:10000

2.7 Primary Cell Culture

2.7.1 Human Adipose-derived Perivascular Stem Cells

Human adipose-derived perivascular stem cells (hAd-PSC; $CD146^{pos}$, $CD34^{neg}$, $CD31^{neg}$, $CD45^{neg}$), isolated from the stromal vascular fraction of human lipoaspirates (Crisan *et al.*, 2008), were gratefully received from Professor Bruno Pèault (MRC Centre for Regenerative Medicine, University of Edinburgh). Technical advice with the handling of these cells was kindly provided by Dr Zaniah Gonzalez Galofre (MRC Centre for Regenerative Medicine, University of Edinburgh).

hAd-PSCs were maintained in expansion media (EM) consisting of DMEM GlutaMAX™-I (Gibco® Thermo Scientific, Runcorn, UK) supplemented with 20% (vol/vol) fetal bovine serum (FBS; Gibco®, Thermo Scientific, Runcorn, UK) and 1% (vol/vol) penicillin-streptomycin (Gibco® Thermo Scientific, Runcorn, UK). Cells were maintained at 37°C with 5% CO_2 using a Heracell™ 150i incubator (Thermo Scientific, Runcorn, UK). Cells were sub-cultivated at a ratio of 1:4. Briefly, cells were washed with sterile PBS and incubated with TrypLE Express dissociation reagent (Gibco® Thermo Scientific, Runcorn, UK) for 2mins at room temperature. Enzyme activity was quenched by the addition of culture media and cells were seeded into fresh EM. Cells were not sub-cultivated beyond passage 10. In the first instance, cells were expanded and frozen at early passage in FBS containing 10% (vol/vol) DMSO (Hybri-Max™; Sigma-Aldrich, Dorset, UK) using a Mr. Frosty™ Freezing Container (Thermo Scientific, Runcorn, UK) containing fresh isopropyl alcohol. Cells were stored in the vapour phase of liquid nitrogen. Frozen cells were rapidly defrosted in a water bath at 37°C and seeded directly into expansion media. After ~16hrs, when the cells had adhered, the media was replaced with fresh media to remove DMSO.

2.7.1.1 *In Vitro* Differentiation

To determine whether hAd-PSCs could differentiate into Leydig-like cells *in vitro*, cells were treated with a cocktail of hormones/growth factors initially described by Ge *et al.* (2006). Briefly, cells were grown to sub-confluence (~70%) in EM (see section 2.7.1) and then switched to a differentiation-inducing medium (DIM) for seven days. DIM consisted of phenol red-free DMEM/F-12 (Gibco® Thermo Scientific, Runcorn, UK) supplemented with 2% FBS (Gibco®, Thermo Scientific, Runcorn, UK), 1%

penicillin-streptomycin (Gibco® Thermo Scientific, Runcorn, UK), 10ng/mL recombinant human platelet-derived growth factor beta (rhPDGFb; Sigma-Aldrich, Dorset, UK), 1ng/mL human luteinising hormone (hLH; Sigma-Aldrich, Dorset, UK), 70ng/mL recombinant human insulin-like growth factor-1 (rhIGF-1 Sigma-Aldrich, Dorset, UK), 1nM 3,3′,5-Triiodo-L-thyronine sodium salt (thyroid hormone, T3; Sigma-Aldrich, Dorset, UK) and 1X human ITS supplement (Sigma-Aldrich, Dorset, UK).

2.8 Statistical Analyses

All statistical analyses were carried out using GraphPad Prism 7.02 software (GraphPad software Inc, San Diego, CA, USA). The distribution of datasets was first assessed using the D'Agostino & Pearson or Shapiro-Wilk normality test, where a *p*-value ≥0.05 represents conformation to a Gaussian distribution (parametric data), and a *p*-value ≤0.05 indicates deviation from the Gaussian distribution (non-parametric data).

Comparisons were made between two groups using either an unpaired, two tailed, *t*-test or a Mann-Whitney *U*-test for parametric and non-parametric data respectively. In each case, a *p*-value ≤0.05 was considered statistically significant.

Differences between more than two groups were identified using either a one-way analysis of variance (ANOVA) or a Kruskal –Wallis test for parametric and non-parametric data respectively. For one-way ANOVA, means were compared between groups using either Dunnet's or Tukey's post hoc analysis. For Kruskal-Wallis, means were compared between groups using Dunn's post hoc analysis. In each case, a *p*-value ≤0.05 was considered statistically significant.

To determine whether inheritance of transgenes conformed to predicted patterns of Mendelian inheritance, the observed genotype ratios were compared to the expected values using a Chi-square test. Significant discrepancy in the observed ratios was indicated by a *p*-value ≤0.05

2.9 Commonly Used Buffers and Solutions

2.9.1 Lysis Buffer –Genomic DNA Extraction

2.9.1.1 Tris Stock Solution

For 1L of Tris stock (1M Tris, pH 8.0), 121.14g Tris base (Sigma-Aldrich, Dorset, UK) was dissolved in 800mL deionised water. After adjusting the pH to 8.0 using concentrated hydrochloric acid (HCl) or sodium hydroxide (NaOH), added dropwise, to decrease or increase pH respectively, the volume was made up to 1L with deionised water. The solution was stored at room temperature.

2.9.1.2 Ethylenediaminetetraacetic Acid (EDTA) Stock Solution

For 1L of EDTA stock (0.5M EDTA, pH 8.0), 146.12g EDTA (Sigma-Aldrich, Dorset, UK) was dissolved in 800mL deionised water. After adjusting the pH to 8.0, the volume was then made up to 1L with deionised water. The solution was stored at room temperature.

2.9.1.3 Tris-EDTA-Tween

Fresh Tris-EDTA-Tween was prepared before use. This was achieved by adding 2.5mL 1M Tris (2.9.1.1), 100µL 0.5M EDTA (2.9.1.2) and 250uL TWEEN® 20 (Sigma-Aldrich, Dorset, UK) to 48mL deionised water. After thorough mixing, the solution was sterile filtered through a 0.22µm syringe filter unit (Merk Millipore, Billerica, MA, USA).

2.9.2 Citrate HIER Buffer

2.9.2.1 Citrate Buffer Stock Solution

For 1L of 10X citrate buffer stock (0.1M citric acid, pH 6.0), 21.01g citric acid monohydrate (Sigma-Aldrich, Dorset, UK) was dissolved in 875mL deionised water. NaOH was added dropwise to achieve a pH of 5.5. The volume was then made up to 1L with deionised water, and further adjusted to pH 6.0. The solution was stored at 4^{o}C.

2.9.2.2 Citrate Buffer Working Solution

Typically, 250mL 1X citrate buffer working solution (10mM citric acid, pH 6.0) was prepared by adding 25mL 0.1M stock (see 2.9.2.1) to 225mL deionised water.

2.9.3 Tris-EDTA HIER Buffer

For 1L of 1X Tris-EDTA buffer (10mM Tris, 1mM EDTA, pH 9.0), 1.21g Tris base (Sigma-Aldrich, Dorset, UK) and 0.37g EDTA (Sigma-Aldrich, Dorset, UK) were dissolved in 900mL deionised water. 500µL Tween® 20 (Sigma-Aldrich, Dorset, UK) was then added before adjusting the final volume to 1L. The solution was stored at 4°C.

2.9.4 Tris-Buffered Saline (TBS)

2.9.4.1 TBS Stock Solution

For 1L of 10X TBS stock (0.2M Tris, 1.5M NaCl, pH 7.6), 24g Tris HCl (Sigma-Aldrich, Dorset, UK), 5.6g Tris base (Sigma-Aldrich, Dorset, UK) and 88g NaCl (Sigma-Aldrich, Dorset, UK) were dissolved in 900mL deionised water and adjusted to pH 7.6 using concentrated HCl or NaOH to decrease or increase pH respectively. The volume was then made up to 1L and the solution stored at 4°C.

2.9.4.2 TBS Working Solution

Generally, 10L of 1X TBS working solution (20mM Tris, 150mM NaCL, pH 7.6) was prepared for communal laboratory use by adding 1L of 10X stock (2.9.4.1) to 9L deionised water.

2.9.5 Phosphate Buffered Saline (PBS)

For 1L of 1X PBS working solution (10mM Na_2HPO_4, 2.7mM KCl, 137mM NaCl, pH 7.4) 5 PBS tablets (Sigma-Aldrich, Dorset, UK) were dissolved in 950mL deionised water. Once dissolved, the volume was adjusted to 1L.

2.9.6 Normal Sera and Blocking Solutions

2.9.6.1 Normal Sera

Normal sera were obtained through the SuRF histology facility from Biosera, Uckfield UK. Normal goat (NGS), chicken (NChS) or horse (NHS) sera were used for immunohistochemistry experiments (see 2.2.3) in which secondary antibodies were raised in the respective species.

2.9.6.2 Blocking Solutions.

For 200mL of blocking solution, 2g bovine serum albumin (BSA; Sigma-Aldrich, Dorset, UK) was dissolved into 140mL of TBS (see 2.9.4.2), 20mL of normal serum was then added and the volume made up to 200mL with TBS. The solution was driven through a 0.22µm syringe filter unit (Merk Millipore, Billerica, MA, USA). Aliquots were stored at -20^{o}C.

3 Dissecting the Paracrine Network in the Ageing Testis

3.1 Introduction

Spermatogenesis and steroidogenesis; the two principal functions of the testis are entirely dependent on the two major somatic cell populations – the Sertoli cells and Leydig cells, respectively. In human males, testicular function declines during the ageing process (Perheentupa and Huhtaniemi, 2009, Gunes *et al.*, 2016). Of particular significance is the reported age-related decrease in Leydig cell androgen production (Morley *et al.*, 1997, Harman *et al.*, 2001, Feldman *et al.*, 2002, Wu *et al.*, 2008, Fabbri *et al.*, 2016) as androgens have been suggested to play a crucial role in supporting lifelong general health in men, with low circulating testosterone linked to an increased risk of developing chronic age-related cardiometabolic diseases (Kupelian *et al.*, 2006, Kupelian *et al.*, 2008, Farrell *et al.*, 2008, Brand *et al.*, 2014, Pye *et al.*, 2014). Therefore, elucidating the mechanism(s) governing the maintenance of testicular function, in particular Leydig cell steroidogenesis, is pertinent to our understanding of male health.

Androgen production is under negative feedback control by the hypothalamic-pituitary-gonad (HPG) axis. Gonadotrophin-releasing hormone (GnRH), from the hypothalamus, stimulates the pituitary to secrete luteinising hormone (LH), which stimulates Leydig cell testosterone production. Increased circulating testosterone then negatively regulates the hypothalamic-pituitary unit to control LH secretion. The nature of this negative feedback loop gives rise to separate etiologies for low testosterone levels observed during ageing/disease. Either a deficit in LH production by the hypothalamic pituitary unit (hypogonadotrophic hypogonadism/secondary hypogonadism) or defective androgen biosynthesis at the level of the Leydig cell, usually accompanied by a compensatory increase in LH and subsequent distortion of the LH/testosterone ratio (hypergonadotrophic hypogonadism/primary hypogonadism). In ageing males, the latter is generally observed although testosterone levels may remain within the normal range due to the compensatory increase in LH (Wu *et al.*, 2008).

Current rodent models for the study of age-related Leydig cell dysfunction are limited. The most widely used model is the naturally-aged brown Norway rat which has provided significant insight into changes occurring in Leydig cells during ageing, including: increased production of reactive oxygen species (ROS), decreased LH-stimulated cAMP signalling, and reduction in the expression and activity of enzymes involved in the conversion of cholesterol to testosterone (Zirkin and Tenover, 2012). However, this model is time consuming and expensive to generate and is accompanied by protracted welfare issues as the animals are generally aged up to 24 months. As such, new models which provide a platform to study the mechanism(s) by which testis/Leydig cell function deteriorates during ageing/disease are required. Ideally, a model permitting the separation of individual components of the system (i.e. other testicular somatic cell populations which may influence Leydig cell function in a paracrine manner) would be valuable in assessing cause and effect.

Genetic models of premature ageing provide an attractive alternative for the expedited study of age-related processes. Indeed, a number of progeroid mouse lines have been generated to date, primarily through disruption of genes required for DNA repair/genomic stability or lamin processing/maintenance of nuclear architecture (Carrero *et al.*, 2016, Kõks *et al.*, 2016). However, the majority have been generated to model monogenic human progeroid syndromes and thus, have limited utility in the study of age-related HPG axis dysfunction. Furthermore, in such models of global gene ablation, it is difficult to separate the contribution of specific tissue/cell types to the observed phenotypes. A study by Chen *et al.* (2009c) reported a premature ageing phenotype in mice deficient for *Cisd2* (CDGSH iron sulphur domain 2), characterised by nerve and muscle degeneration, osteopenia, reduced body weight and premature death. Conversely, over expression of *Cisd2* in transgenic mice has been reported to extend healthy lifespan and delay ageing; protecting against age-associated mitochondrial dysfunction (Wu *et al.*, 2012). CISD2 is a redox active protein localised to the endoplasmic reticulum (ER) and is thought to be important for the maintenance of ER and mitochondrial structure/function (Wiley *et al.*, 2013).

3.1.1 Hypothesis & Aims

It is hypothesised herein that *Cisd2*-deficient mice will have a testicular phenotype resembling that of the ageing testis and thus be useful for the expedited study of age-related testicular dysfunction. The overall aim of this chapter was to determine the utility of novel models of premature ageing to dissect the process of age-related testicular degeneration. Firstly, a novel knockout-first conditional allele of a previously reported premature-ageing model driven by *Cisd2* deficiency (*Cisd2*$^{tm1a(EUCOMM)Wtsi}$) was validated and the testicular phenotype characterised and compared to that of naturally aged mice at 18-months of age. Next, Cre/*LoxP* technology was used to delete *Cisd2* from specific testicular cell populations in *Cisd2*$^{tm1c(EUCOMM)Wtsi}$ mice to determine the contribution of individual cell types in the control/support of Leydig cell function during the ageing process.

3.2 Results

3.2.1 Effect of Constitutive CISD2-Knockout in the Adult Testis

3.2.1.1 Validation of the *Cisd2*$^{tm1a(EUCOMM)Wtsi}$ Allele

To establish whether CISD2-deficient mice would provide a suitable platform for the study of age-related testicular dysfunction, the absence of CISD2 protein in *Cisd2*$^{tm1a(EUCOMM)Wtsi}$ mice was first confirmed. Wild-type (WT), heterozygous (HET) and homozygous (KO) mice were generated as described section 2.1.2.1.1. Genotyping primers were designed around the synthetic cassette and used to identify WT, HET and KO animals (Figure 3.1A). Chi-squared analysis of offspring derived from heterozygous crossings confirmed inheritance of WT and KO alleles conform to predicted Mendelian ratios (Figure 3.1B). Western blotting on a panel of tissues including liver and testis (Figure 3.1C) confirmed the absence of CISD2 protein in KO mice. Furthermore, growth curves from WT, HET and KO mice (Figure 3.1D) revealed CISD2-deficient animals were significantly lighter from 8 weeks of age compared to WT animals, consistent with previous reports of *Cisd2* disruption (Chen *et al.*, 2009c). Taken together, these data confirm that the knockout-first *Cisd2*$^{tm1a(EUCOMM)Wtsi}$ allele functions as expected and that CISD2 loss associated with this allele is functionally significant.

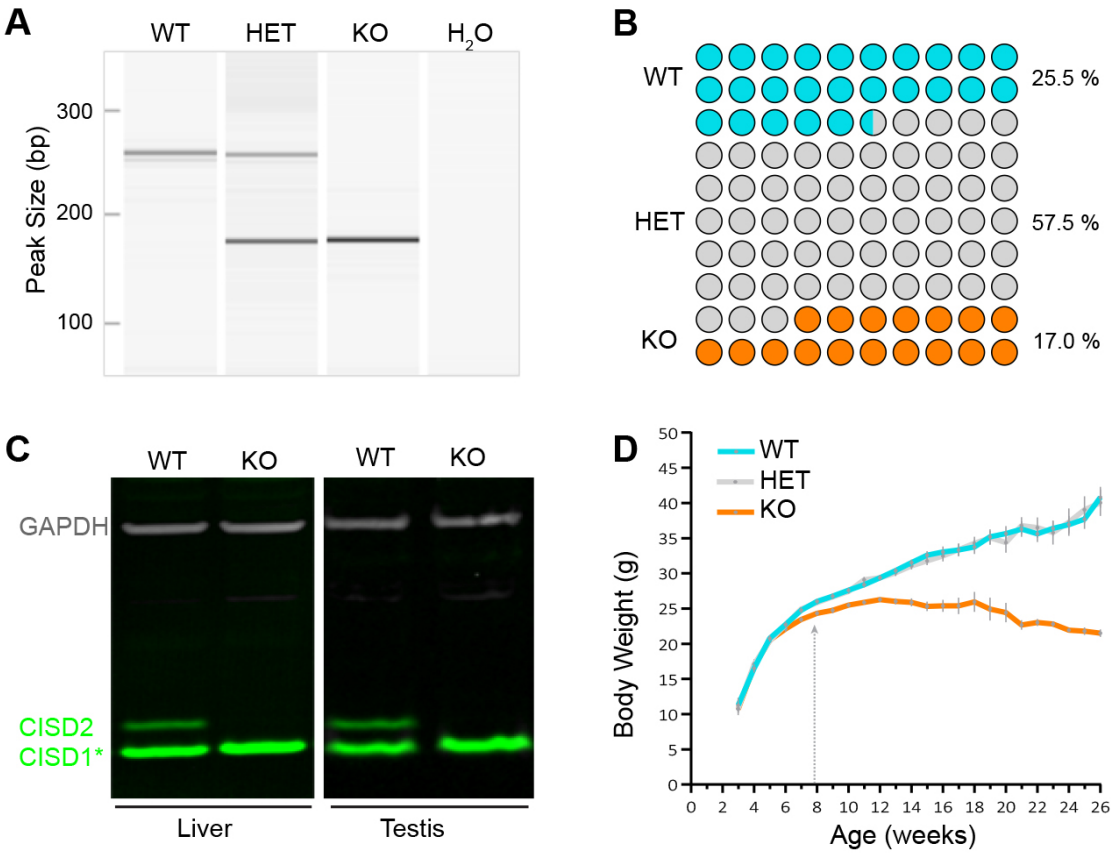


Figure 3.1 Validation of the Knockout-first Cisd2 Allele. (**A**) PCR analysis of genomic DNA isolated from ear clips of wild-type (WT; 257bp), heterozygous (HET; 257 and 178bp) and homozygous (KO; 178bp) mice. (**B**) Inheritance of WT and KO alleles in offspring derived from heterozygous crossings conformed to predicted Mendelian ratios (X^2; $p = 0.4224$). (**C**) Western blot analysis of liver and testis confirmed CISD2 (CDGSH iron sulphur domain 2) protein is absent in KO mice. *The lower band is predicted to be CISD1 (CDGSH iron sulphur domain 1) based on size and sequence homology in the region of the protein that the antibody was raised against. (**D**) Growth curves of WT, HET and KO mice revealed CISD2-deficient animals were significantly lighter from 8 weeks of age compared to WT animals (WT vs. KO individual *t*-tests; $p = 0.006823$ at 8wks, $p = 0.000419$ at 26 weeks. n = ≥5 animals per genotype per time point). Values are mean ± S.E.M.

3.2.1.2 Testicular Atrophy in CISD2-deficient Mice

Having validated ablation of CISD2 in KO animals, the impact of CISD2 deficiency on testicular architecture was next assessed. No overt dysmorphology of the reproductive tract was noted although testes and seminal vesicles appeared slightly smaller in KO animals at 6-months of age (Figure 3.2A). In addition, a striking reduction in epididymal fat pad mass was observed in the KO mice, in line with the previously reported requirement of CISD2 for normal adipogenesis (Wang *et al.*,

2014). Indeed, testis weight was significantly lower in 6-month old KO mice compared to age-matched WT contols (Figure 3.2B). Although testicular histology was largely normal in KO mice, with abundant interstitial Leydig cells and full spermatogenesis occurring within seminiferous tubules, occasional degenerating tubules were noted, similar to observations of the 18-month old naturally aged testis (Figure 3.2C).

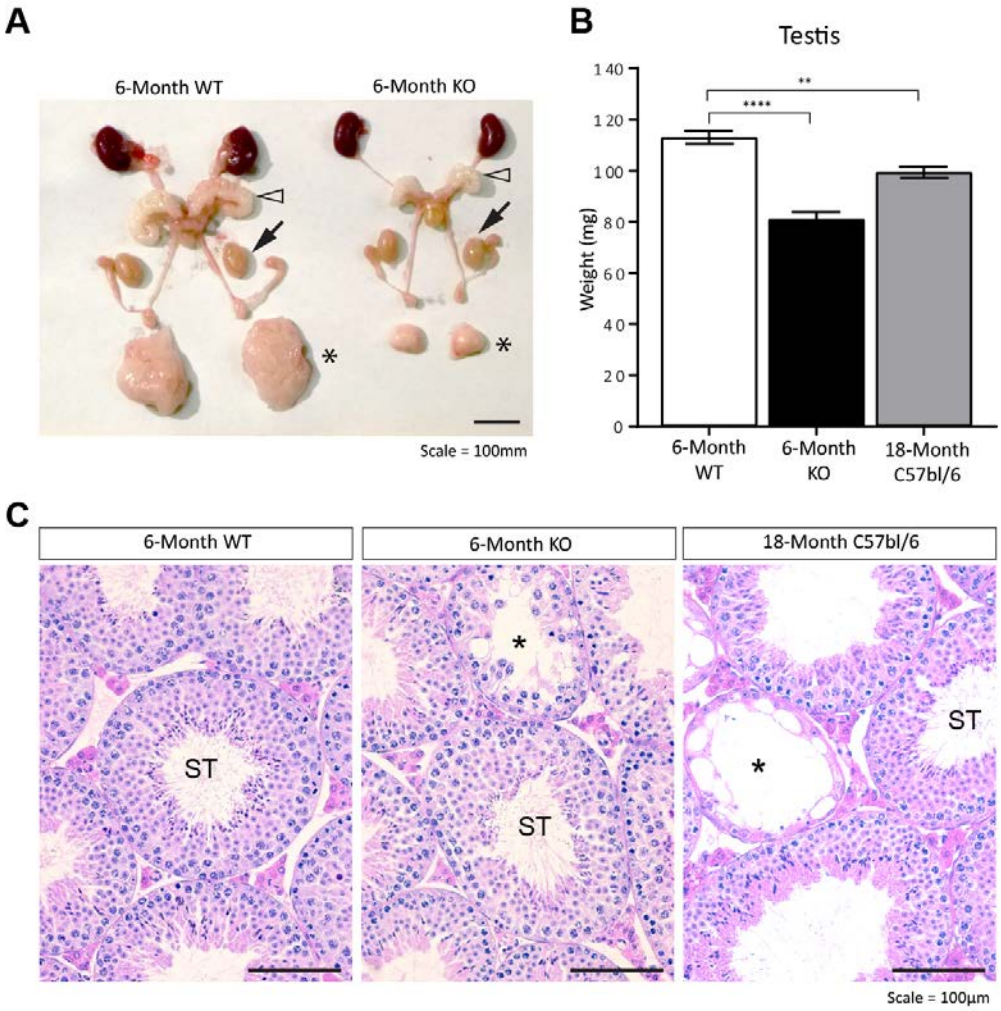


Figure 3.2 Testis Weight and Architecture is Altered in CISD2-deficient Mice. (A) Representative reproductive tracts from 6-month old wild-type (WT) and knockout (KO) mice. Open arrowheads = seminal vesicle, closed arrows = testis. Note the striking reduction in epididymal fat pad mass (asterisks). Scale = 10mm. (B) A significant reduction in testis weight was observed in KO mice at 6-months of age. Testis weights of naturally aged mice were also reduced at 18 months of age (1-way ANOVA; *p* = <0.0001; n = 8-10). Error bars = S.E.M. (C) H & E stained testis sections from WT and KO mice at 6-months of age and naturally aged mice at 18-months of age. In each case abundant interstitial Leydig cells and seminiferous tubules (ST) with full spermatogenesis were present. However, occasional degenerating tubules (asterisks) were noted in the KO testis, consistent with observations of the naturally aged testis. Scale = 100µm.

Given that the structure and function of the seminiferous tubules is entirely dependent on the Sertoli cell population (Rebourcet *et al.*, 2014a, Rebourcet *et al.*, 2014b), the tubular degeneration in the KO testis may be explained by a deficit in this cell population. Immuno-reactivity of the Sertoli cell marker SOX9 (SRY-Box 9) was observed, as expected, around the periphery of the seminiferous tubules in WT, KO and naturally aged testes (Figure 3.3A). Stereological analysis revealed that there are significantly fewer Sertoli cells in the KO testis at 6-months of age (Figure 3.3B). This decrease in the Sertoli cell population was associated with a significant reduction in the diameter of the seminiferous tubules (Figure 3.3C) and a consequent reduction in the epididymal sperm reserve in KO mice (Figure 3.3D). Taken together, these observations suggest CISD2 loss results in premature testicular atrophy at 6-months of age, consistent with that observed in 18-month old naturally aged mice.

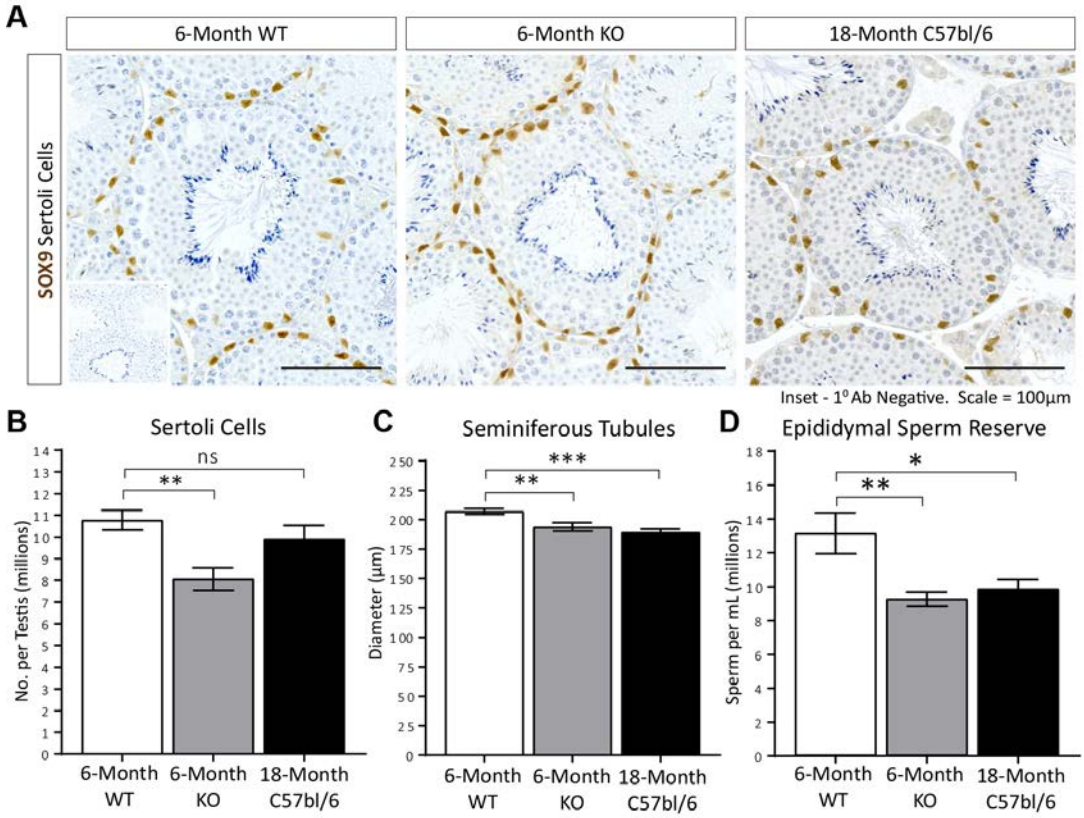


Figure 3.3. Sertoli Cell Number, Seminiferous Tubule Diameter and Epididymal Sperm Reserves are Reduced in CISD2-deficient Mice. (**A**) Representative chromogenic immunostaining of the Sertoli cell marker SOX9 (SRY-Box 9) in wild-type (WT), knockout (KO) and naturally aged testes. Sertoli cell number (**B**), seminiferous tubule diameter (**C**) and epididymal sperm reserves were significantly reduced in KO testes at 6-months of age consistent with changes occurring in the naturally aged testis (1-way ANOVA; p = 0.0034, 0.0011 and 0.0062 respectively, n = 5-8). Error bars = S.E.M.

3.2.1.3 Leydig Cells are Dysfunctional in CISD2-deficient Mice

Ageing is associated with a reduction in Leydig cell steroidogenesis (Chen *et al.*, 2015). Plasma testosterone levels along with seminal vesicle weight (as a biomarker of peripheral androgen action) were reduced in the KO mice compared to WT controls at 6-months of age (Figure 3.4A and B), indicating a perturbation in Leydig cell testosterone biosynthesis in the absence of CISD2. Conversely, seminal vesicle weight was significantly increased in naturally aged mice (Figure 3.4B). Although circulating luteinising hormone levels were not significantly elevated in the KO mice (Figure 3.4C), the LH:T ratio was significantly higher (Figure 3.4D), suggesting that the reduction in testosterone production is likely due to primary testicular dysfunction rather than secondary to dysfunction of the hypothalamic-pituitary unit.

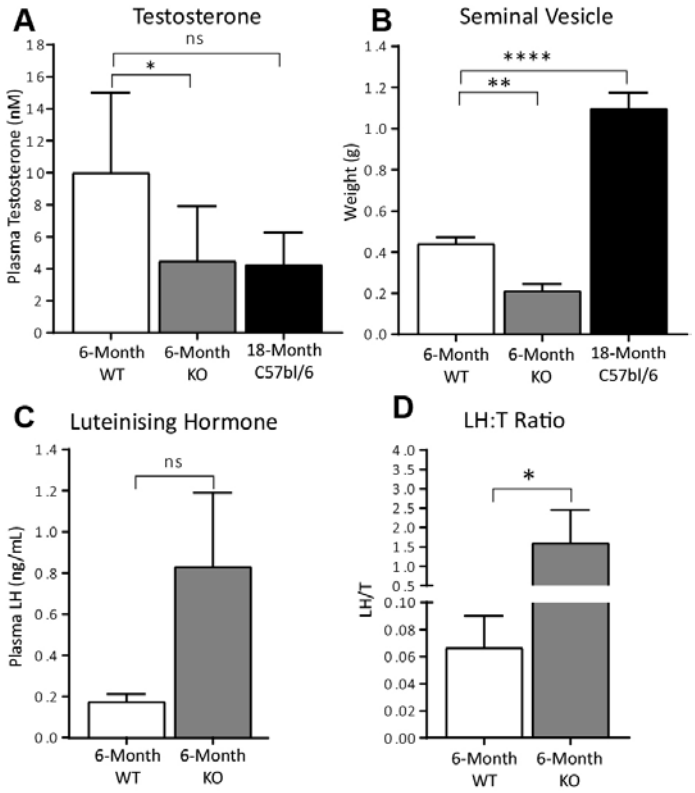


Figure 3.4 Leydig Cell Dysfunction Resembling Compensated Leydig cell Failure in CISD2-deficient Mice. (**A**) Circulating testosterone was reduced in knockout (KO) mice at 6-months of age (Kruskal-Wallis; p = 0.0158, n =5-12). (**B**) Seminal vesicle weight (a biomarker of peripheral androgen action) was significantly reduced in KO animals at 6-months of age converse to the naturally aged animals (1-way ANOVA; p = <0.0001, n =5-10). (**D**) A significant increase in the luteinising hormone:testosterone ratio (LH/T) was observed in the KO mice suggesting observed reduction in testosterone is due to dysfunction of CISD2-deficient Leydig cells (Mann-Whitney *U*-test; p = 0.0393, n= 5-9). Error bars = S.E.M.

To further probe the basis for the observed reduction in testosterone in CISD2-deficient mice (Figure 3.4A), Leydig cell number and the mRNA expression of genes involved in steroidogenesis were assessed in KO testes at six-months of age. As expected, immuno-reactivity of the Leydig cell marker HSD3B (hydroxysteroid dehydrogenase 3-beta) was observed in the interstitial compartment in WT, KO and naturally aged testes (Figure 3.5A). However, a significant reduction in Leydig cell number was observed in both 6-month old KO and naturally aged testes (Figure 3.5A). Furthermore, mRNA expression of key Leydig cell-expressed genes, required for the conversion of cholesterol to testosterone, were significantly decreased in KO testes (Figure 3.5B). Despite a reduction in Leydig cell number in the aged testis (Figure 3.5A), mRNA expression of steroidogenic genes remained largely stable suggesting that reduced expression in the KO testes reflects impaired function rather than simply a decrease in Leydig cell number.

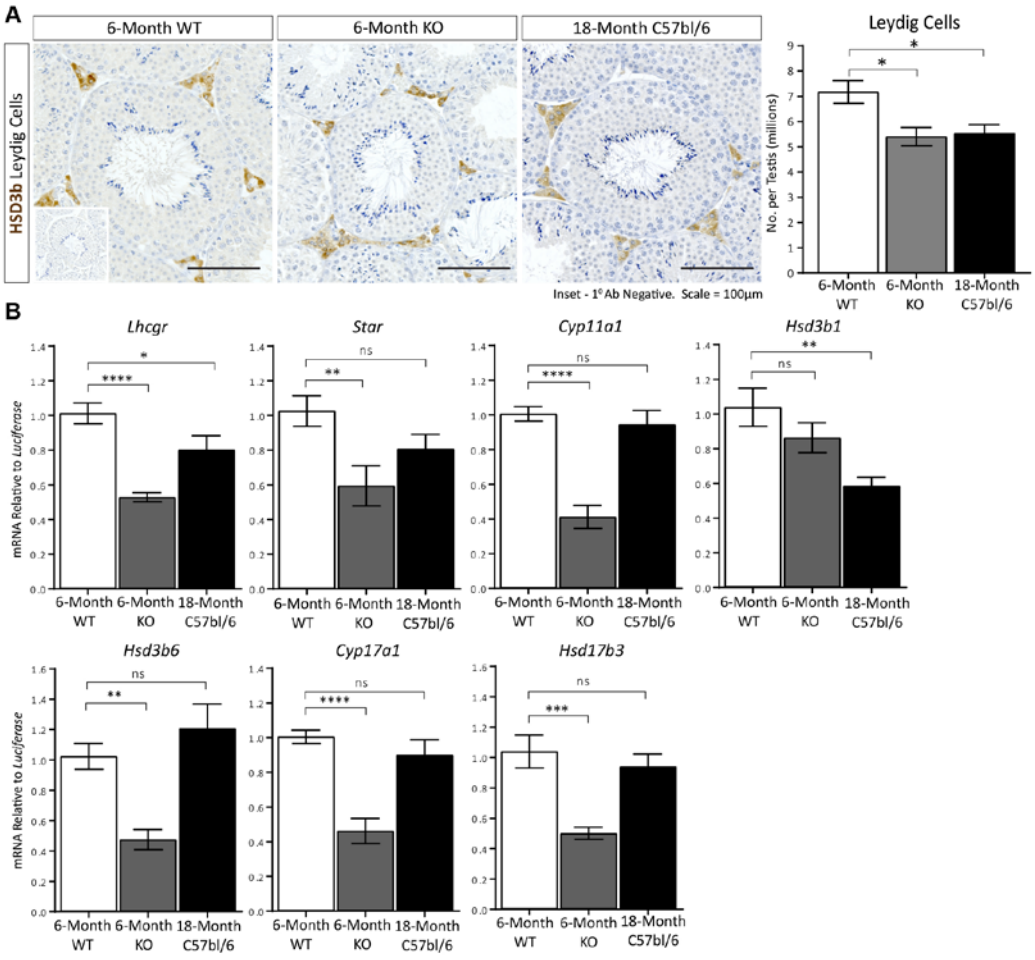


Figure 3.5 Leydig Cell Number and Function is Impacted in CISD2-deficient Mice at 6-months of Age. *Legend on following page*

Figure 3.5 **Leydig Cell Number and Function are Altered in CISD2-deficient Mice at 6-months of Age.** *Figure on preceding page* (**A**) Representative chromogenic immunostaining of the Leydig cell marker hydroxysteroid dehydrogenase 3-beta (HSD3B) in wild-type (WT), knockout (KO) and naturally aged testes. Leydig cell number was reduced in both KO and naturally aged testes (1-way ANOVA; p = 0.0086; n = 5-8). (**B**) mRNA expression of genes involved in Leydig cell testosterone biosynthesis was reduced in the KO testis at 6-months of age whereas expression was largely unchanged in the naturally aged testis with the exception of *Lhcgr* and *Hsd3b1* (1-way ANOVA). Luteinising hormone/chorionic gonadotropin receptor (*Lhcgr*), p = <0.0001; steroidogenic acute regulatory protein (*Star*), p = 0.0172; P450 cholesterol side-chain cleavage enzyme (*Cyp11a1*), p = <0.0001; hydroxysteroid dehydrogenase 3-beta type 1 (*Hsd3b1*), p = 0.0045; hydroxysteroid dehydrogenase 3-beta type 6 (*Hsd3b6*), p = 0.0004; 17α-hydroxylase, 17,20-lyase (*Cyp17a1*) p = <0.0001; hydroxysteroid dehydrogenase 17-beta type 3 (*Hsd17b3*), p = 0.0003; n = 8). Error bars = S.E.M.

3.2.2 Effect of Cell-specific *Cisd2*-KO in the Adult Testis

To better understand how the testicular microenvironment is affected in CISD2-KO mice, a conditional *Cisd2* allele (*Cisd2*$^{tm1c(EUCOMM)Wtsi}$) was used to disrupt *Cisd2* in Leydig and Sertoli cells separately.

3.2.2.1 *Pdgfrb*-Cre Targets Adult Leydig Stem/Progenitor Cells in the Fetal Testis

The utility of *Pdgfrb*-Cre mice for the targeting of the adult Leydig cell population was assessed using a red fluorescent protein (RFP) Cre reporter allele (*Gt(ROSA)*$^{26Sortm14(CAG-tdTomato)Hze}$). During pubertal development adult Leydig cells develop from a pool of peritubular and/or perivascular stem/progenitor cells which is established during fetal life (Siril Ariyaratne *et al.*, 2000, Mendis-Handagama and Ariyaratne, 2001, O'Shaughnessy *et al.*, 2002b, Davidoff *et al.*, 2004, Ge *et al.*, 2005, Ge *et al.*, 2006, Chen *et al.*, 2009b, Kilcoyne *et al.*, 2014). Dual fluorogenic immunostaining revealed widespread expression of PDGFRB in the interstitial compartment of the mouse testis at e18.5 with Cre-mediated RFP expression noted in spindle-shaped interstitial/peritubular cells (Figure 3.6B). Co-localisation of RFP and the putative stem/progenitor marker COUP-TFII (Kilcoyne *et al.*, 2014) was observed in a proportion of spindle-shaped peritubular cells (Figure 3.6C) suggesting the *Pdgfrb*-Cre line may be useful for conditional gene ablation in the adult Leydig cell population.

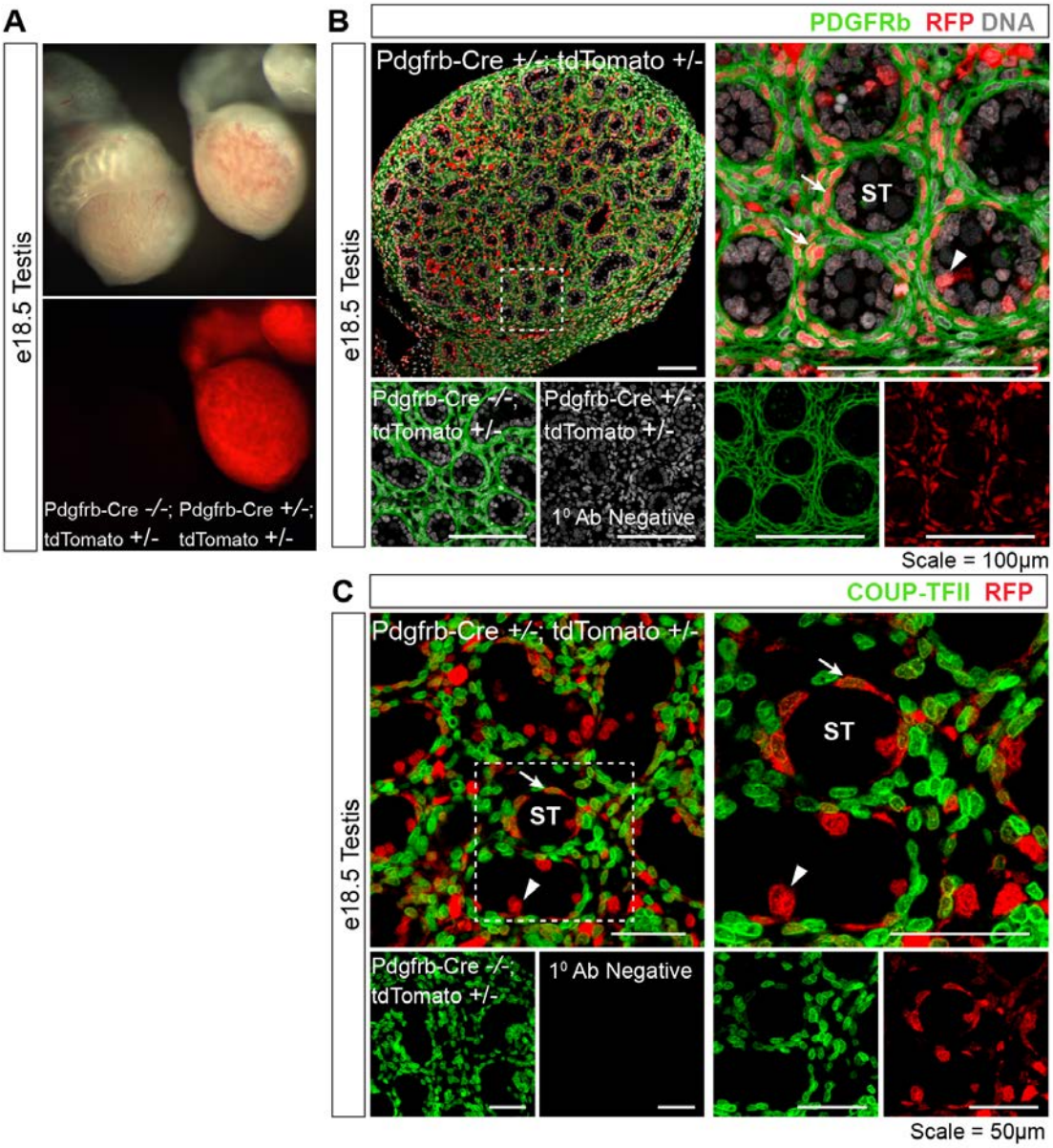


Figure 3.6 Pdgfrb-Cre Targets Adult Leydig Cell Stem/Progenitor Cells in the Fetal Testis. (**A**) tdTomato red fluorescent protein (RFP) expression was observed in the testes of td-Tomato Cre-reporter mice at embryonic day (e)18.5. (**B**) Dual fluorogenic immunostaining revealed widespread expression of platelet-derived growth factor receptor beta (PDGFRB) in the interstitial compartment of the fetal testis. Cre-mediated RFP expression was noted in spindle-shaped interstitial/peritubular cells (arrows); some RFP expression was also noted in a number of Sertoli cells (arrowheads). (**C**) Co-localisation of RFP and the putative stem/progenitor marker chicken ovalbumin upstream promoter transcription factor-II (COUP-TFII) was observed in a proportion of spindle-shaped peritubular cells (arrows) which are hypothesised to give rise to the adult Leydig cell population. ST = seminiferous tubule. Arrowhead = Sertoli cell.

3.2.2.2 Generation of Leydig and Sertoli Cell-specific *Cisd2*-KO Mice

Having demonstrated that the *Pdgfrb*-Cre line targets adult Leydig stem/progenitor cells in the fetal testis, targeting of the adult Leydig cells was next confirmed. Dual fluorescent immunohistochemistry for RFP and HSD3B revealed co-localisation in approximately 55 per cent of Leydig cells in the testis at postnatal day (d) 50 (Figure 3.7A). RFP expression was also observed in peritubular and perivascular cells as well as in a small sub-population of Sertoli cells. Specificity of the *Amh*-Cre line was also confirmed in RFP reporter mice by dual fluorescent immunohistochemistry for RFP and SOX9. As expected, Cre expression was confined to testicular Sertoli cells as evidenced by the pattern of RFP positive staining, characteristic of Sertoli cell cytoplasm, in cells positive for the Sertoli cell nuclear marker SOX9 (Figure 3.7B). PCR amplification of genomic DNA isolated from the testes of *Prdgfrb*-Cre$^{-/-}$; *Cisd2*$^{tm1c/tm1c}$ (WT), and *Prdgfrb*-Cre$^{+/-}$; *Cisd2*$^{tm1c/tm1c}$ (LC-KO) and *Amh*-Cre$^{-/-}$; *Cisd2*$^{tm1c/tm1c}$ (WT) and *Amh*-Cre$^{+/-}$; *Cisd2*$^{tm1c/tm1c}$ (SC-KO) mice demonstrated recombination of the conditional *Cisd2* allele upon exposure to either Cre-recombinase (Figure 3.7C and D). In contrast to the phenotype observed in global-KO animals in which body weight was significantly reduced from 8-weeks of age, body weight was maintained in LC-KO and SC-KO animals (Figure 3.8A). In fact, a slight increase in body weight was noted in LC-KO animals at 6-months of age.

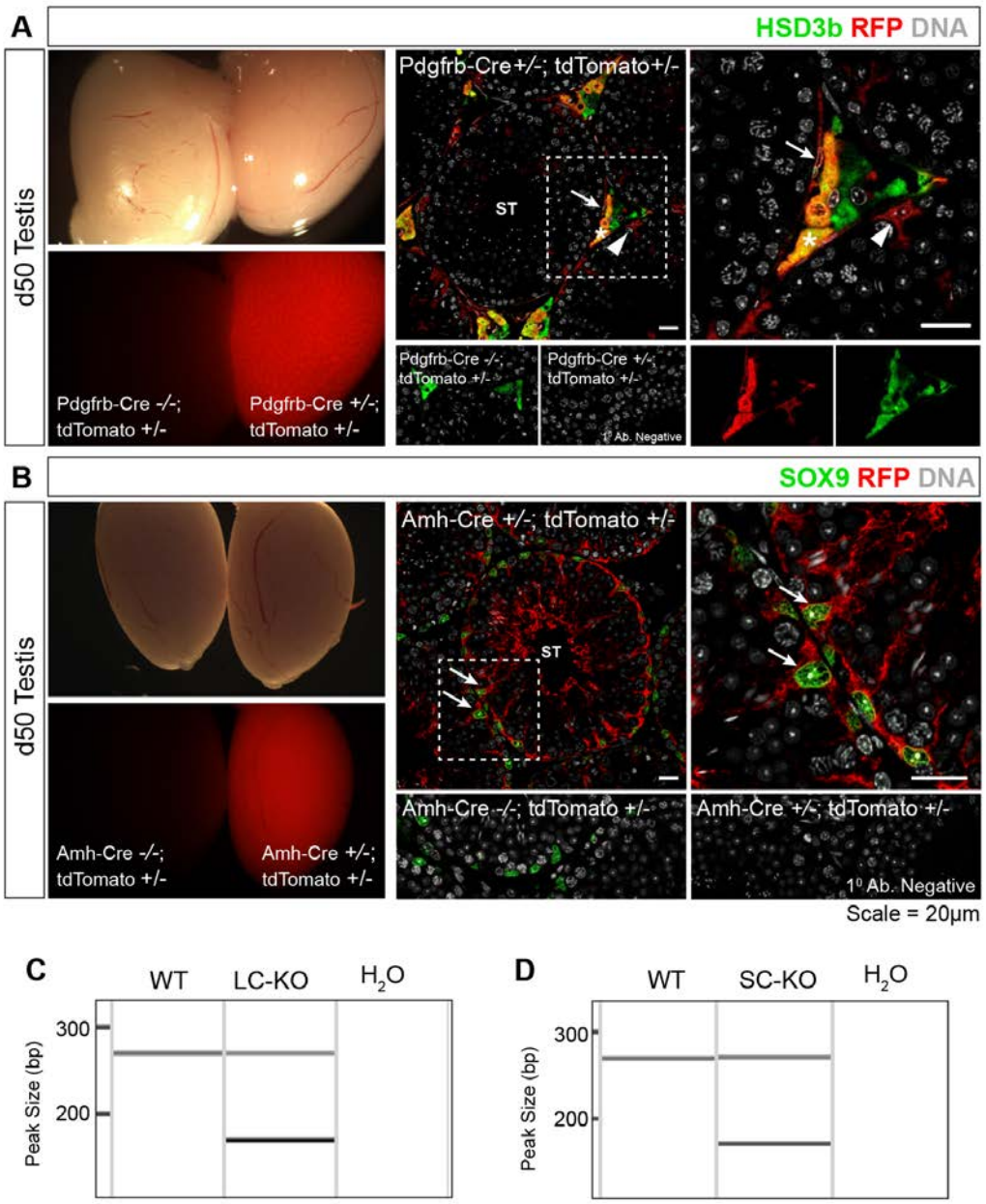


Figure 3.7 Generation of Leydig and Sertoli Cell-Specific *Cisd2*-KO Mice. (**A**) Analysis of *Pdgfrb*-Cre activity in the testis of young adult tdTomato Cre-reporter mice. Co-immunostaining of red fluorescent protein (RFP) with the Leydig cell (LC) marker hydroxysteroid dehydrogenase 3-beta (HSD3B; asterisks) showed that this Cre line is active in approximately 55 ± 3.9 % (S.E.M) of adult Leydig cells. Expression was also observed in the peritubular cells (arrows) as well as in a small proportion of Sertoli cells (closed arrowheads). (**B**) Confirmation of Sertoli cell-specific targeting using *Amh*-Cre. Co-immunostaining of RFP with the Sertoli cell marker SRY box-9 (SOX9) showed RFP expression was restricted to Sertoli cells. PCR analysis of testicular genomic DNA confirmed the conditional *Cisd2* allele is recombined upon exposure to either (**C**) *Pdgfrb*-Cre (Leydig cell knockout; LC-KO) or (**D**) *Amh*-Cre (Sertoli cell knockout; SC-KO). Wild-type (WT) = 268bp, Knockout (KO) = 174bp.

3.2.2.3 Disruption of *Cisd2* either in Leydig or Sertoli Cells does not Induce Testicular Degeneration

As testicular atrophy accompanied by decreased Sertoli cell number, seminiferous tubule diameter and epididymal sperm reserves is observed in global-KO testes at six months of age (see section 3.2.1.2), it was next asked whether this phenotype is recapitulated when *Cisd2* is disrupted either in Leydig cells or Sertoli cells alone. Testis weight was normal in both LC-KO and SC-KO animals (Figure 3.8B), with no histological evidence of tubular degeneration (Figure 3.8C) as noted in constitutive *Cisd2*-KO animals. Additionally, Sertoli cell number, tubule diameter and epididymal sperm reserves were similar between LC-KO, SC-KO and their respective controls (Figure 3.9A-D). Taken together, these observations suggest that neither Leydig cells nor Sertoli cells alone are responsible for the degenerative testicular phenotype observed in constitutive *Cisd2*-KO animals.

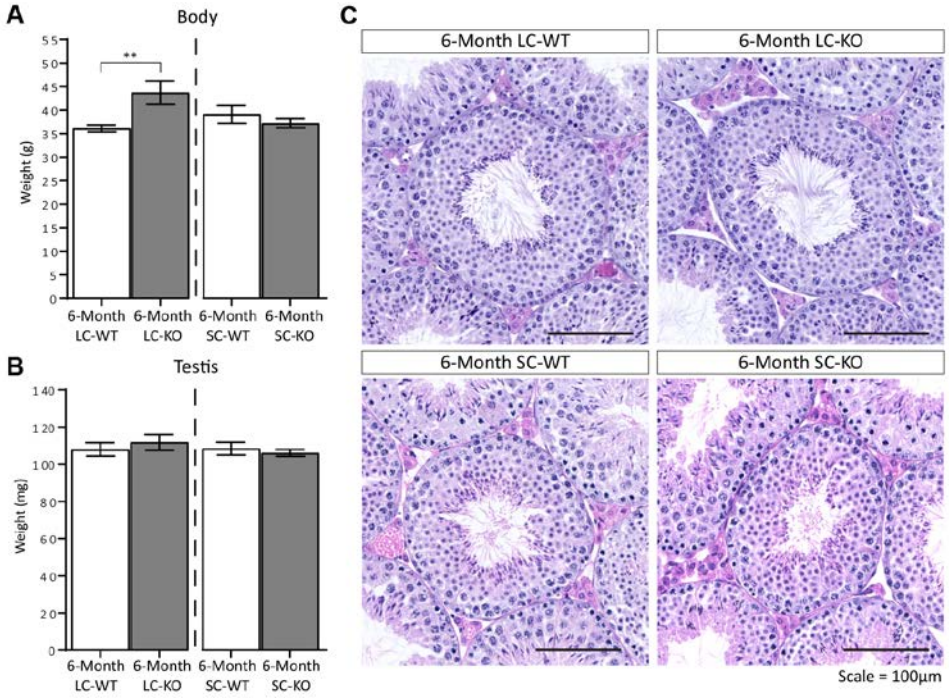


Figure 3.8 Testicular Architecture is Unaltered when *Cisd2* is Disrupted either in Leydig or Sertoli Cells. (**A**) Body weight was not reduced in Leydig cell knockout (LC-KO; left) or Sertoli cell knockout (SC-KO; right) animals. In fact, a slight increase in weight was observed in LC-KO animals at 6-months of age (unpaired *t*-test; $p = 0.0096$). (**B**) No difference in testis weight was observed between control and LC-KO or SC-KO mice (unpaired *t*-test). n = 7-8. Error bars = S.E.M. (**C**) Representative H & E stained testis sections of LC-KO (top) and SC-KO (bottom) mice and 6-months of age. Testicular architecture remained normal when *Cisd2* is disrupted either in Leydig or Sertoli cells.

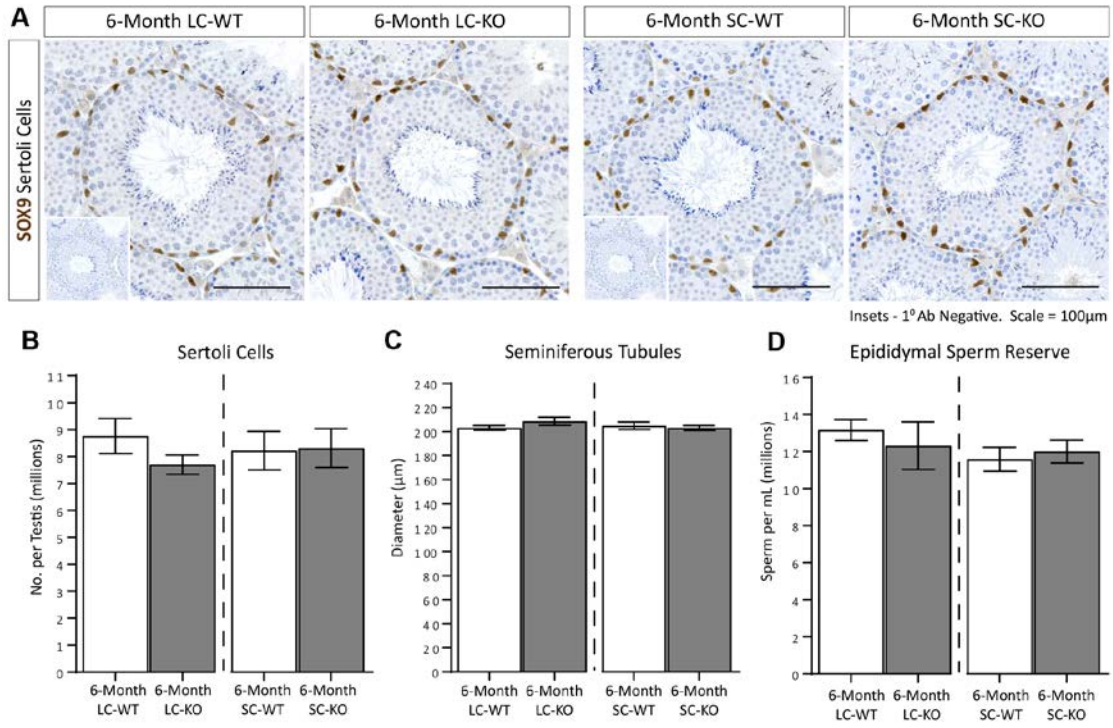


Figure 3.9 Sertoli Cell Number, Seminiferous Tubule Diameter and Epididymal Sperm Reserves are Maintained When *Cisd2* is Disrupted in Leydig or Sertoli Cells. (**A**) Representative chromogenic immunostaining of the Sertoli cell marker Sry box-9 (SOX9) in 6-month old Leydig cell knockout (LC-KO) and Sertoli cell knockout (SC-KO) testes and their respective controls. In contrast to global KO and naturally aged animals, no reduction in (**B**) Sertoli cell number, (**C**) seminiferous tubule diameter or (**D**) epididymal sperm reserves was noted in the LC-KO or SC-KO mice at 6-months of age (unpaired *t*-tests). n = 7-9. Error bars = S.E.M.

In addition to endocrine regulation by pituitary-derived luteinising hormone, Leydig cell steroidogenesis is thought to also be subject to regulation by a paracrine network within the testis (Saez, 1994). As such, Leydig cell steroidogenic function was assessed in both conditional *Cisd2*-KO models to determine whether the Leydig cell dysfunction observed in constitutive CISD2-KO animals is a direct consequence of C*isd2* loss from the Leydig cell population, or due to indirect effects of Sertoli cell *Cisd2* disruption. Interestingly, circulating testosterone levels were normal both in LC-KO and in SC-KO animals at 6-months of age (Figure 3.10).To test whether maximal Leydig cell testosterone production was altered in either conditional models, hCG-stimulated testosterone was measured. No difference in maximal testosterone production was observed when *Cisd2* was disrupted in Leydig cells or in Sertoli cells (Figure 3.10). Additionally, in contrast to global-KO animals, Leydig cell number

(Figure 3.11A) and steroidogenic mRNA expression (Figure 3.11B) were also normal in both models compared to their respective controls. Together, these data suggest that Leydig cell steroidogenic function is maintained in LC-KO and SC-KO animals. These results demonstrate that the mechanism(s) underlying primary testicular failure observed in the prematurely aged global-KO animals cannot be assigned to specific dysfunction of the Leydig or Sertoli cell populations upon *Cisd2* disruption.

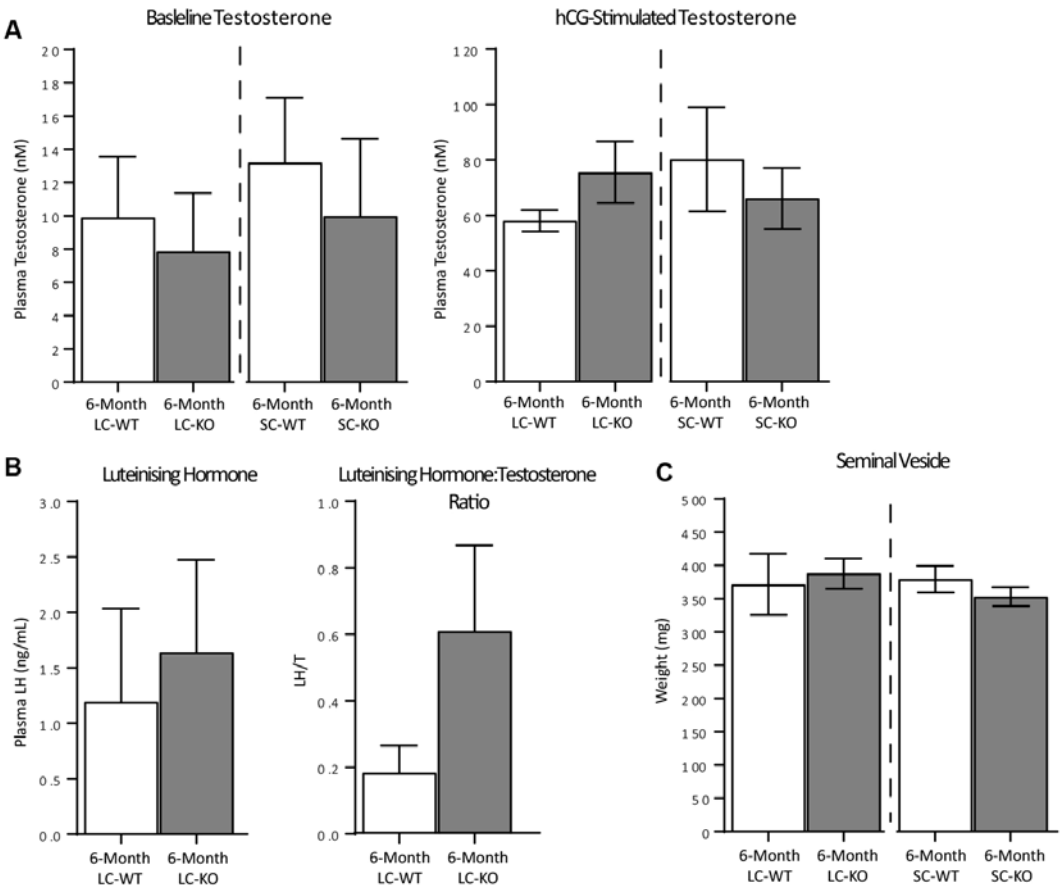


Figure 3.10 Circulating Testosterone is Maintained when *Cisd2* is Disrupted either in Leydig cells or in Sertoli cells. Converse to the global KO animals, plasma testosterone (**A**) remained normal when *Cisd2* was disrupted in Leydig cells (LC-KO; unpaired *t*-test, n = 6-7) or Sertoli cells (SC-KO; Mann Whitney *U*-test n = 10-11). (**B**) No difference in circulating luteinising hormone (LH) or the LH:T ratio was observed in LC-KO mice at 6-months of age (Mann Whitney *U*-test n = 6-7). (**C**) No difference in seminal vesicle weight was observed in LC-KO or SC-KO mice (unpaired *t*-tests n = 7-8). Error bars = S.E.M.

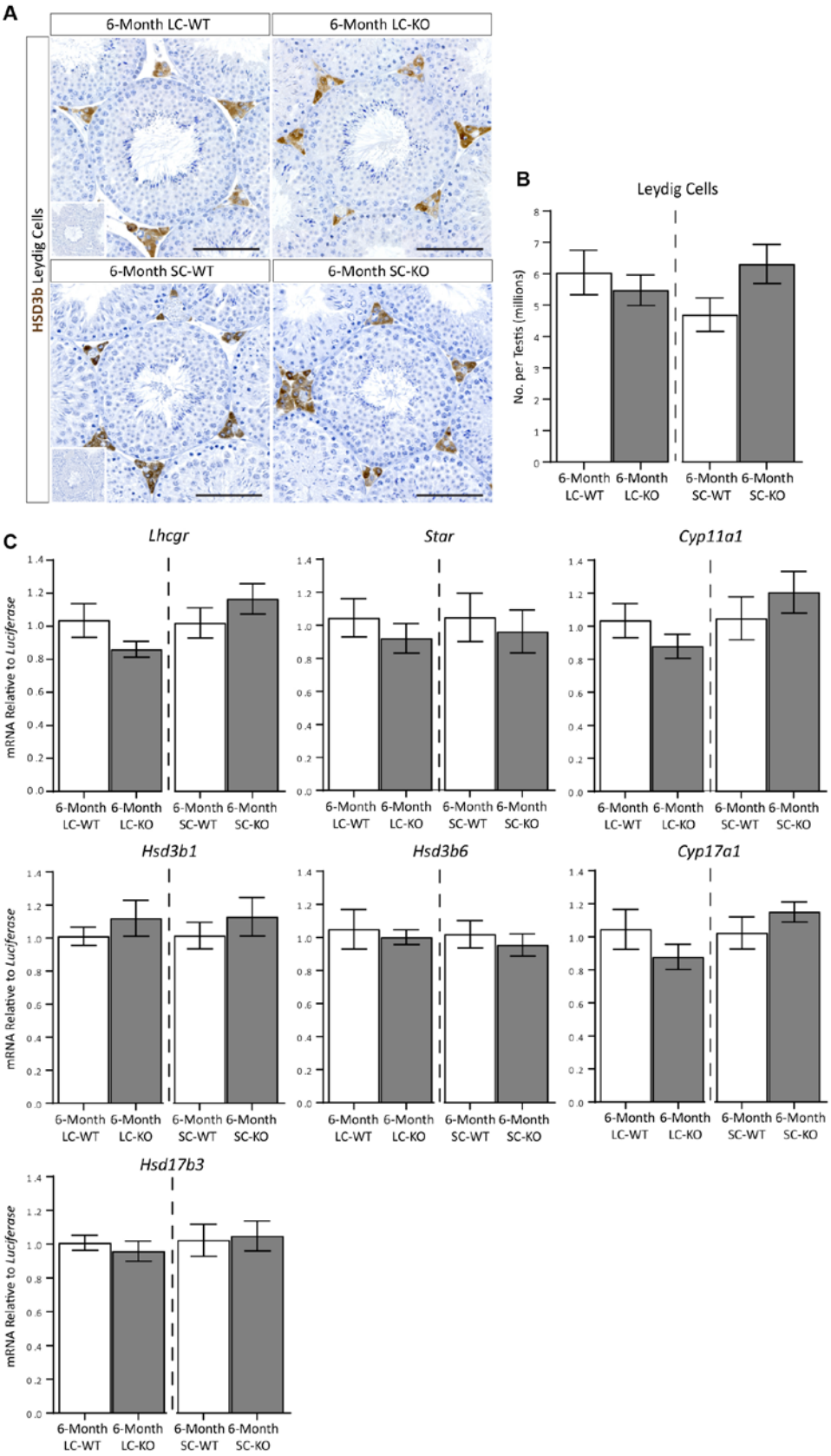


Figure 3.11. Leydig Cell Number and Function are Maintained when *Cisd2* is Disrupted either in Leydig Cells or Sertoli Cells. *Legend on following page.*

Figure 3.11. Leydig Cell Number and Function are Maintained when *Cisd2* is Disrupted either in Leydig Cells or Sertoli Cells. *Figure on preceding page.* (**A**) Representative chromogenic immunostaining of the Leydig cell marker hydroxysteroid dehydrogenase (HSD3B) in 6-month old Leydig cell knockout (LC-KO) and Sertoli cell knockout (SC-KO) testes and their respective controls. (**B**) Stereological analysis revealed normal Leydig cell number in both LC-KO and SC-KO testes (unpaired *t*-tests). n = 7-8. Error bars = S.E.M. (**C**) No difference in the expression of steroidogenic mRNAs was observed in the LC-KO testis (unpaired *t*-tests). n = 6-8. Error bars = S.E.M.

3.3 Discussion

Ageing in men is accompanied by a decline in testicular function. Of particular importance is the reported age-related decrease in the endocrine function of the testis (e.g. androgen production) (Morley *et al.*, 1997, Harman *et al.*, 2001, Feldman *et al.*, 2002, Wu *et al.*, 2008, Fabbri *et al.*, 2016), as an inverse relationship between testosterone and cardiometabolic disease risk has been suggested (Brand *et al.*, 2014, Pye *et al.*, 2014, Farrell *et al.*, 2008, Kupelian *et al.*, 2006, Kupelian *et al.*, 2008). However, the cause-consequence relationship between androgens, ageing and disease is not entirely clear. Specifically, the precise mechanism(s) by which Leydig cell testosterone production becomes compromised, leading to age-related primary hypogonadism, remains to be fully established. The studies presented in this chapter utilised a series of novel mouse models of premature ageing to begin to dissect the process of age-related testicular degeneration.

Age-related testicular atrophy is well documented both in humans (Johnson *et al.*, 1986, Paniagua *et al.*, 1991, Mahmoud *et al.*, 2003, Yang *et al.*, 2011, Jiang *et al.*, 2013) and in rodents (Gosden *et al.*, 1982, Wang *et al.*, 1993, Wright *et al.*, 1993, Levy *et al.*, 1999, Morales *et al.*, 2004, Xia *et al.*, 2011). Histomorphometric analyses of CISD2-KO animals reveal a testicular phenotype resembling age-related atrophy, occurring 12 months earlier than naturally in aged mice. In men testicular volume declines with age (Handelsman and Staraj, 1985, Johnson *et al.*, 1986, Mahmoud *et al.*, 2003, Yang *et al.*, 2011) and is linked to a reduction in Sertoli cell number and/or function, involution of the seminiferous tubules and diminished spermatogenesis (Johnson *et al.*, 1984b, Johnson *et al.*, 1986, Johnson *et al.*, 1990, Paniagua *et al.*, 1991, Jiang *et al.*, 2013). This is consistent with the reduction in testis weight, seminiferous

tubule diameter, Sertoli cell number and epididymal sperm reserves observed in both CISD2-KO and naturally aged mice.

Testicular endocrine function also deteriorates during the ageing process. Decreased testosterone production may be attributed to either dysfunction of the hypothalamic-pituitary unit, resulting in decreased LH secretion or, due to the diminished capacity of aged Leydig cells to produce testosterone, often accompanied by increased circulating LH (Wu *et al.*, 2008). In fact, both testosterone and LH measurement are required to inform on Leydig cell function, as circulating testosterone may remain within the normal range owing to compensatory LH. A reduction in circulating testosterone was noted in CISD2-KO mice at 6-months of age. Although LH is not significantly higher, the LH/testosterone ratio is significantly increased in CISD2-deficient mice, indicative of primary Leydig cell dysfunction (Tajar *et al.*, 2010). Interestingly, there is no difference in testosterone levels between WT mice at 6 and 18-months. Unfortunately, due to technical and time limitations, circulating LH was not measured in naturally aged mice, as such compensation by the hypothalamic pituitary unit to maintain circulating testosterone cannot be ruled out. Previous studies of HPG-axis function in ageing mice report conflicting findings. For example, testosterone is reported to decrease in ageing mice due to reduced frequency and amplitude of LH pulses (Bronson and Desjardins, 1977, Coquelin and Desjardins, 1982). However, these studies were carried out using 30-month old CBF_1 mice, whereas analyses herein were carried out on plasma from 18-month old C57BL/6 mice; thus, either the age or the strain difference may explain this discrepancy. In support of the latter, plasma testosterone levels are reported to remain stable in C57BL/6 up to 31-months of age (Eleftheriou and Lucas, 1974, Finch *et al.*, 1977) suggesting that the C57BL/6 strain may be refractory to age-related Leydig cell dysfunction. When Nelson *et al.* (1975) considered healthy and diseased aged C57BL/6 mice separately, a reduction in circulating testosterone was observed in diseased mice. However, the cross-sectional nature of this study prevents any interpretation of causality (i.e. whether low testosterone levels drive disease progression or *vice versa*). Additionally, circulating gonadotrophins were not measured, thus the authors are unable to conclude whether the hypogonadism noted in the diseased animals is primary or secondary in nature.

Testosterone deficiency may be attributed to reduced number and/or function of Leydig cells. Early studies reported up to a forty four percent reduction in Leydig cell number in the ageing human testis, accompanied by a two-fold increase in LH to maintain circulating testosterone levels (Kaler and Neaves, 1978, Neaves *et al.*, 1984, Neaves *et al.*, 1985). However, a more recent study reported decreased numbers of Sertoli, but not Leydig cells in the ageing human testis (Petersen *et al.*, 2015). In addition to the relatively small sample sizes of the above studies, these discrepancies may be due to differences in methodologies employed to quantify Leydig cells. Consistent with the findings of Petersen *et al.* (2015), Leydig cell number is reported to be maintained in aged Brown Norway rats, whereas steroidogenic function is reduced due, in part, to reduced expression of genes required for testosterone biosynthesis (Wang *et al.*, 1993, Chen *et al.*, 1994). In the studies presented herein, a reduction in the number of Leydig cells accompanied by reduced mRNA expression of genes encoding the luteinising hormone receptor and steroidogenic enzymes in CISD2-KO animals at six months of age was noted. Interestingly, although a similar reduction in Leydig cell number was observed in the naturally aged animals, Leydig cell steroidogenic function is maintained. This strongly suggests that the decreased testosterone production in CISD2-KO mice is due to impaired Leydig cell function rather than simply a deficit in cell number. In line with previous studies by Chen *et al.* (2009c) and Wang *et al.* (2014), the CISD2-KO mice described herein have a marked reduction in adipose tissue which may confound analyses of reproductive function. Indeed, resection of the epididymal white adipose tissue depot has been shown to have a negative impact on spermatogenesis, however circulating testosterone and LH are unaffected (Chu *et al.*, 2010). Thus, CISD2-KO mice represent a novel model of age-related primary hypogonadism.

Multiple changes intrinsic to aged Leydig cells including decreased number and sensitivity of LH receptors (Chen *et al.*, 2002) as well as decreased expression and activity of proteins required for the conversion of cholesterol to testosterone (Culty *et al.*, 2002, Luo *et al.*, 1996), have been suggested to play a role in their reduced efficiency to produce testosterone. Damage to steroidogenic machinery as a result of perturbation in the balance between the generation of reactive oxygen species (ROS), and their neutralisation by antioxidants, is hypothesised to underlie the decreased

testosterone production by aged Leydig cells (Chen and Zirkin, 1999, Chen *et al.*, 2001, Diemer *et al.*, 2003, Luo *et al.*, 2006, Beattie *et al.*, 2013). However, these data are primarily derived from *in vitro* experiments and, thus, fail to recapitulate the *in vivo* environment, including paracrine interactions, which may influence Leydig cell function. Studies by Wiley *et al.* (2013) demonstrated that CISD2-loss results in a pro-oxidative intracellular environment which may explain the reduction in testosterone observed in CISD2-KO mice. However, in prematurely ageing mitochondrial DNA mutator mice (Trifunovic *et al.*, 2004), despite a seven-fold increase in Leydig cell superoxide production, testosterone biosynthesis is unaffected thus, increased ROS may not be directly toxic to Leydig cells (Shabalina *et al.*, 2015). This would be consistent with maintenance of Leydig cell function in LC-KO mice and, suggests that alterations in factors extrinsic to Leydig cells may impair steroidogenesis in aged testes. Indeed, while Chen *et al.* (1996) reported that EDS-mediated elimination of hypo-functional Leydig cells and their subsequent regeneration in the ageing rat testis restored circulating testosterone to levels to that of young animals, it was later reported that this elevation in testosterone is not maintained long term (Chen *et al.*, 2015). This suggests that alterations in the microenvironment of the aged testis in which the Leydig cells reside may play an important role in age-associated Leydig cell dysfunction.

Sertoli cell number declines in ageing men (Johnson *et al.*, 1984b, Jiang *et al.*, 2013, Petersen *et al.*, 2015). Interestingly, age-related Sertoli cell dysfunction has been reported to precede reductions in Leydig cell testosterone production (Haji *et al.*, 1994), raising the possibility that dysfunction of the Sertoli cell population may alter Leydig cell function. For example, treatment of Sertoli-Leydig cell co-cultures with FSH has been reported to increase LHCGR expression and testosterone production (Benahmed *et al.*, 1985, Perrard-Sapori *et al.*, 1986, Saez *et al.*, 1989). As such, it was hypothesised herein, that conditional *Cisd2* disruption in Sertoli cells may result in testicular atrophy and primary hypogonadism as observed in constitutive CISD2-KO mice. However, no such phenotype was observed in SC-KO animals up to 6-months of age. Unfortunately, due to technical and time limitations, circulating LH was not measured in SC-KO mice which would further inform on Leydig cell function (i.e. an increase in LH may maintain testosterone levels).

While old animals may be considered the simplest rodent models of ageing; significant time, cost and welfare implications, concomitant with the generation of naturally aged animals, limits their practicality. Furthermore, ageing represents a multifactorial series of complex changes across multiple systems; making it difficult to assign cell/tissue specific contributions to age-related dysfunction. Although mouse models of accelerated ageing may not fully model the natural ageing process, they have been employed as alternatives to shed light on the mechanisms underpinning degenerative processes associated with ageing (Kõks *et al.*, 2016). The testicular phenotype in constitutive CISD2-KO mice closely resembles that of the ageing testis, rendering this line a useful tool for the expedited study of testicular ageing. Furthermore, in contrast to naturally aged mice, the hormonal profile of CISD2-KO animals may better reflect the status of the HPG-axis in ageing men thus providing a useful resource to test novel therapeutic interventions aimed at reversing age-related primary hypogonadism. Based on observations of the conditional *Cisd2*-KO models, it is suggested that disruption to the testicular microenvironment in which Leydig cells reside may play a significant role in age-associated Leydig cell dysfunction.

4 The Role of Leukemia Inhibitory Factor Receptor Signalling in Testicular Development and Function

4.1 Introduction

Both spermatogenesis and steroidogenesis are subject to tight regulation at endocrine and paracrine levels. In addition to negative feedback control of testicular function by the hypothalamic-pituitary-gonadal (HPG) axis, cross-talk between different cell types within the testis is also required to maintain spermatogenesis and steroidogenesis (Gnessi *et al.*, 1997, Schlatt *et al.*, 1997). For example; Leydig cell-derived androgens, signalling *via* androgen receptors in Sertoli cells and peritubular myoid cells, are essential for the maintenance of spermatogenesis (De Gendt *et al.*, 2004, Welsh *et al.*, 2009a, O'Shaughnessy *et al.*, 2010b, Willems *et al.*, 2015) whilst Sertoli cells, peritubular myoid cells and testicular macrophages have been shown to support Leydig cell development and steroidogenesis (Cohen *et al.*, 1996, De Gendt *et al.*, 2005, Welsh *et al.*, 2012, Rebourcet *et al.*, 2014a, Rebourcet *et al.*, 2014b, DeFalco *et al.*, 2015). However, the full extent of the paracrine network which supports testicular function remains to be established. Identification of paracrine factors and/or mechanisms that regulate testicular function will be of benefit to the development of novel treatments for infertility and hypogonadism as well as for male contraceptive strategies.

Locally produced growth factors and cytokines have been suggested to play a role in the regulation of normal testicular development and function (Gnessi *et al.*, 1997, Hedger and Meinhardt, 2003). One such example is leukemia inhibitory factor (LIF); a pleiotropic cytokine belonging to the interleukin-6 family of cytokines, most often noted for its role in maintaining the balance between stem cell proliferation and differentiation (Heinrich *et al.*, 1998). LIF signalling is mediated by a heterodimeric receptor complex consisting of the leukemia inhibitory factor receptor (LIFR, also known as gp190), which binds LIF, and the signal transducing gp130 subunit common to the IL-6 family members (Gearing *et al.*, 1991, Gearing *et al.*, 1992, Ip *et al.*, 1992). Interestingly, mRNA expression of IL-6 type cytokines, including *LIF*, is increased in

the ageing human and is associated with muscular and thymic atrophy (Sempowski *et al.*, 2000, Reardon *et al.*, 2001), possibly implicating LIF/LIFR signalling in age-related tissue dysfunction. In rodents, expression of both LIF and LIFR, as well as the gp130 signal transducer, has been detected in the fetal and adult testis, suggesting LIF/LIFR signalling may play a role in normal testicular development and function (Jenab and Morris, 1998, Piquet-Pellorce *et al.*, 2000, Molyneaux *et al.*, 2003, Dorval-Coiffec *et al.*, 2005). Peritubular myoid cells have been identified as the principal source of LIF within the rat testis and, given the anatomical location of these cells, LIF has been hypothesised to be a paracrine regulator of both the tubular and interstitial compartments (Piquet-Pellorce *et al.*, 2000). Interestingly, LIF-deficient males are reported to be fertile (Stewart *et al.*, 1992) whereas complete knockout of the LIFR results in perinatal death due to pleiotropic defects including neurological and metabolic disturbances (Ware *et al.*, 1995). This suggests that, whilst alternative cytokines of the IL6-type family may be able to substitute for an absence of LIF, LIFR-signalling *per se*, is essential for survival. Whilst LIFR is expressed by somatic Sertoli cells, Leydig cells, peritubular myoid cells and macrophages; spermatogonia have been speculated to be the main target of LIFR signalling within the rat testis based on *in vitro* binding assays with biotinylated LIF and immunohistochemical detection of LIFR in testis sections (Dorval-Coiffec *et al.*, 2005). However, the precise role(s) of testicular LIFR signalling remains to be established.

4.1.1 Hypothesis & Aims

As outlined above, LIF/LIFR may be an important paracrine regulator of testicular function. Therefore, it is hypothesised herein that genetic ablation of *Lifr* will have significant impacts on testis development and function. The overall aim of this chapter was thus to definitively identify role(s) for LIFR signalling in the testis *in vivo*. To this end, cell-specific LIFR deficient mice were generated and the testicular phenotype analysed in order to dissect out and characterise the role of LIFR signalling in the development and function of the testis. Firstly, to determine whether LIFR signalling is required for prenatal testis development, a novel *Lifr* knockout allele (*Lifr*$^{tm1b(EUCOMM)Hmgu}$) was validated and the impact of complete LIFR-loss on testicular structure was assessed in new-born mice at postnatal day 0. A conditional *Lifr* allele

(*Lifr*$^{tm1c(EUCOMM)Hmgu}$) was then used to generate testis cell-specific *Lifr*-knockout mice to identify potential role(s) for LIFR signalling in the postnatal/adult testis.

4.2 Results

4.2.1 LIFR is Not Required for Prenatal Testicular Development

4.2.1.1 Validation of the *Lifr*$^{tm1b(EUCOMM)Hmgu}$ Allele

To determine whether there is a requirement for LIFR signalling in prenatal testis development, the absence of LIFR protein in new-born *Lifr*$^{tm1b(EUCOMM)Hmgu}$ mice was first validated. Wild-type (WT), heterozygous (HET) and homozygous (KO) mice were generated as described in section 2.1.2.1.2. Primers were designed to detect the synthetic targeting cassette (see section 2.1.2 Figure 2.2) and used to amplify genomic DNA isolated from tail tip biopsies to identify WT, HET and KO animals (Figure 4.1A). Western blotting analysis of neonatal whole brain protein extracts revealed that LIFR protein expression was completely abolished in homozygous *Lifr*$^{tm1b(EUCOMM)Hmgu}$ mice (Figure 4.1B). At weaning, on postnatal day (d) 21, a significant deviation from the expected Mendelian genotype ratios was noted (Figure 4.1C), consistent with previous reports of *Lifr*-KO mice (Ware *et al.*, 1995), indicating that the *Lifr*$^{tm1b(EUCOMM)Hmgu}$ allele is a true loss of function allele.

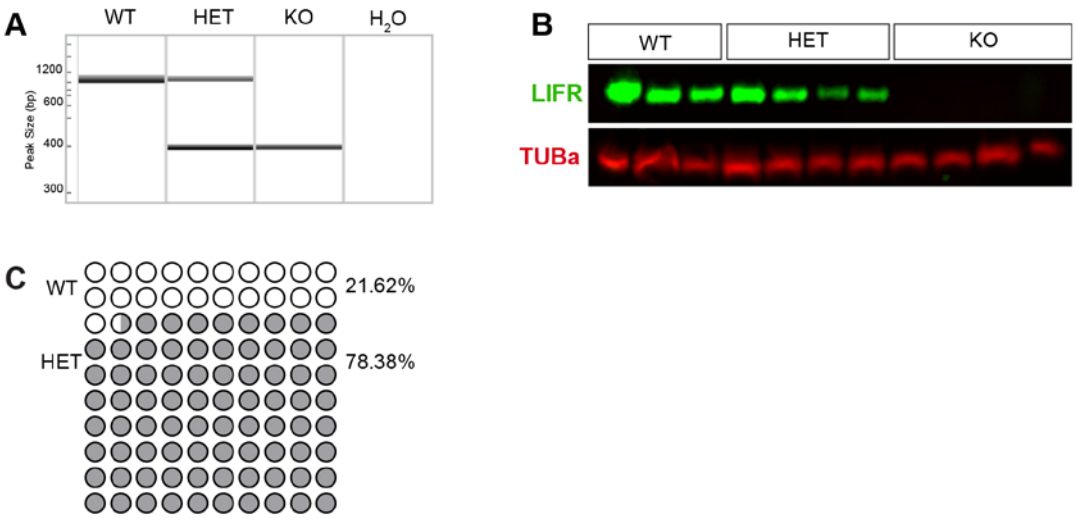


Figure 4.1 Confirmation of LIFR Ablation in *Lifr*$^{tm1b(EUCOMM)Hmgu}$ Mice. *Legend on following page.*

Figure 4.1 Confirmation of LIFR Ablation in *Lifr*$^{tm1b(EUCOMM)Hmgu}$ Mice. *Figure on preceding page.* (**A**) Representative PCR analysis of genomic DNA isolated from tail-tip biopsies of neonatal mice identified wild-type (WT; 1150bp), heterozygous (HET; 1150 and 406bp) and homozygous (KO; 406bp) animals. (**B**) Western blotting analysis of neonatal brain tissue homogenates confirmed leukemia inhibitory factor receptor (LIFR) protein expression was abolished in KO animals. Tubulin-alpha (TUBA) was used as a loading control (**C**) Transgene inheritance in offspring derived from heterozygous matings based on genomic PCR of DNA isolated from ear-clip biopsies collected at weaning. Chi-squared analysis revealed a significant deviation from the expected Mendelian ratios (X^2; p = <0.0001).

4.2.1.2 Testicular Histology in Neonatal Constitutive LIFR-KO Mice

Having confirmed the absence of LIFR protein expression in *Lifr*$^{tm1b(EUCOMM)Hmgu}$ mice (see section 4.2.1.1), the impact of LIFR ablation on the neonatal testis was next assessed. Histological analysis revealed that testicular architecture was normal in LIFR-deficient animals, with fully formed seminiferous cords containing abundant spermatogonia (Figure 4.2A). No differences in the immuno-localisation of Sry box-9 (SOX9; Sertoli cells), mouse vasa homologue (DDX4; germ cells) and hydroxysteroid dehydrogenase 3-beta (HSD3B; Leydig cells) were observed in the testes of WT, HET or KO animals (Figure 4.2B). This suggests that establishment of the Sertoli, germ and fetal Leydig cell populations, and the structural arrangement of these cells in the testis occurs normally in the absence of LIFR.

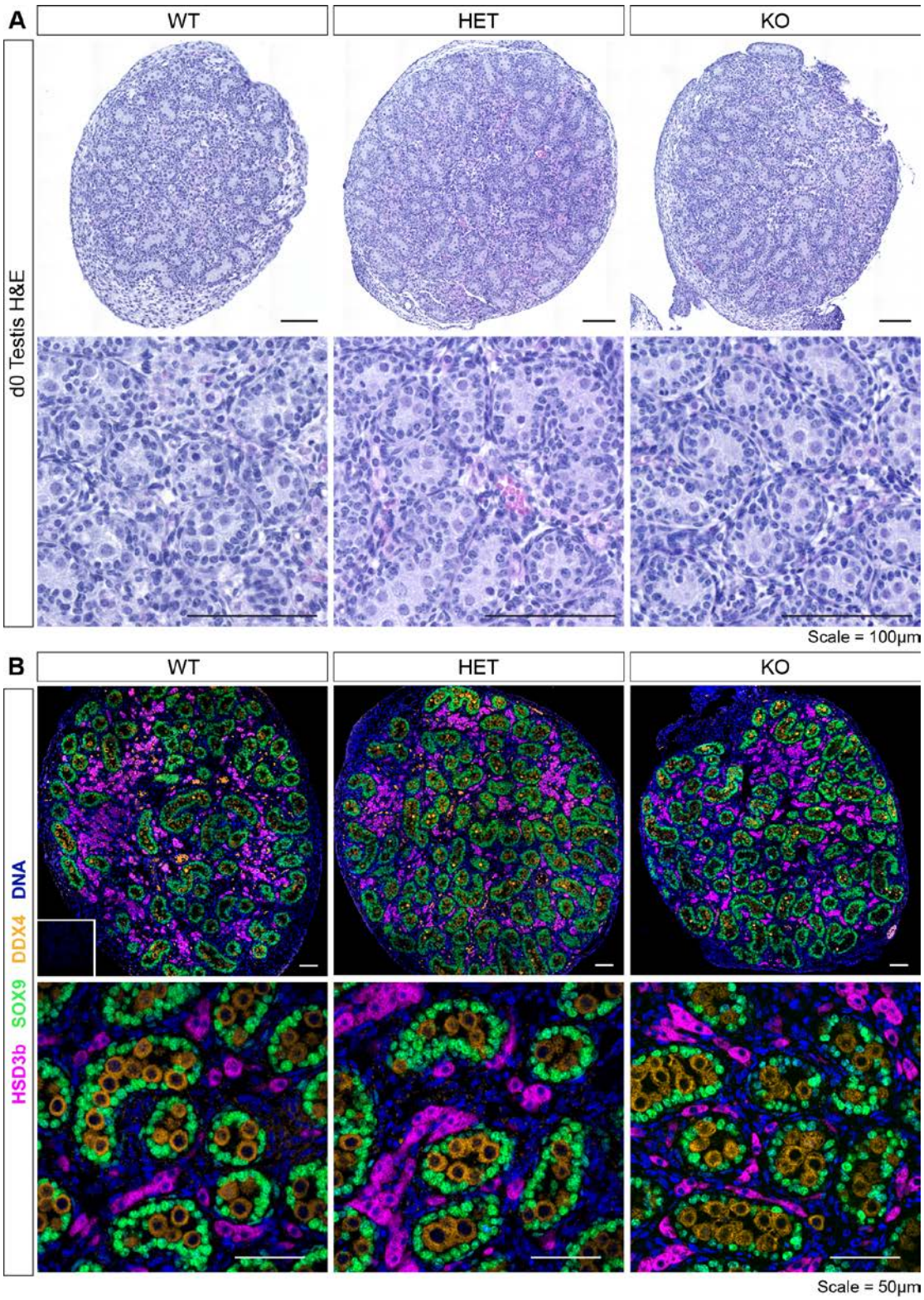


Figure 4.2 Testicular Histology is Normal in LIFR-deficient Mice. *Legend on following page*

Figure 4.2 Testicular Histology is Normal in LIFR-deficient Mice *Figure on preceding page.* (**A**) Representative H&E stained testis sections from wild-type (WT), heterozygous (HET) and homozygous (KO) mice at post-natal day 0. Testes appeared morphologically normal, with fully formed seminiferous cords containing abundant spermatogonia. n = 3-5, scale = 100µm. (**B**) Immunostaining for hydroxysteroid dehydrogenase 3-beta (HSD3B; magenta), Sry box-9 (SOX9; green) and mouse vasa homologue (DDX4; gold) confirmed the presence of fetal Leydig cells, Sertoli cells and spermatogonia respectively. No obvious difference was noted between WT, HET and KO testes. Inset = primary antibody negative control. n = 3-5, scale = 50µm.

4.2.2 LIFR is Required in Sertoli Cells but not Germ Cells to Maintain Normal Spermatogenic Function

To circumvent the lethality observed in constitutive LIFR deficient animals, a conditional variant of the *Lifr* allele (*Lifr*$^{tm1c(EUCOMM)Hmgu}$; see section 2.1.2.2.2) was used to generate cell-specific LIFR knock-outs in order to identify potential role(s) for LIFR signalling in the development/function of the adult testis.

4.2.2.1 Generation of Germ Cell and Sertoli Cell specific *Lifr*-KO Mice

Lifr was selectively disrupted separately in germ cells (GC) and Sertoli cells (SC) or both (SC+GC-KO) using the well-established *Stra8*-Cre (Sadate-Ngatchou *et al.*, 2008) and *Amh*-Cre (Lecureuil *et al.*, 2002) lines (GC-KO and SC-KO respectively). PCR amplification of genomic DNA isolated from the testes of *Stra8*-Cre$^{+/-}$; *Lifr*$^{wt/tm1c}$ and *Amh*-Cre$^{+/-}$; *Lifr*$^{wt/tm1c}$ mice demonstrated recombination of genomic DNA and conversion of the conditional *Lifr*^{tm1c} to the null *Lifr*^{tm1d} allele upon exposure to either Cre-recombinase (Figure 4.3A and B). Furthermore, PCR analysis of testicular cDNA, using primers flanking the critical exon, revealed the presence of a mutant *Lifr* transcript following Cre-mediated recombination of *Lifr* gDNA. This confirmed previous reports that *Lifr* mRNA is expressed both in germ cells and in Sertoli cells (Figure 4.3C and D respectively). Interestingly, Western blot analysis of whole adult testis protein extracts revealed a significant reduction of LIFR protein in SC-KO but not GC-KO testes (Figure 4.3E and F, respectively). This suggests that, although *Lifr* mRNA is expressed in the germ cell population, the Sertoli cell population may be the major target of LIFR signalling within the seminiferous tubule.

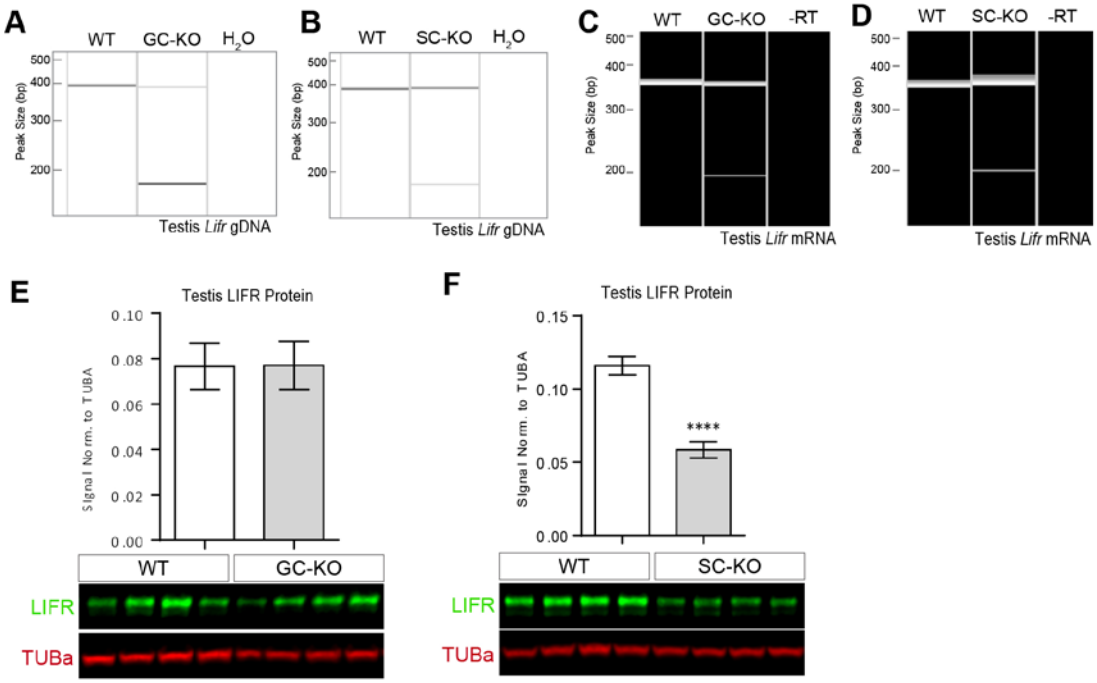


Figure 4.3 Generation of Germ Cell and Sertoli Cell *Lifr*-KO Mice. Representative PCR analysis of testicular genomic DNA from (**A**) *Stra8-Cre*$^{-/-}$*; Lifr*$^{wt/tm1c}$ and *Stra8-Cre*$^{+/-}$*; Lifr*$^{wt/tm1c}$ and (**B**) *Amh-Cre*$^{-/-}$*; Lifr*$^{wt/tm1c}$ and *Amh-Cre*$^{+/-}$*; Lifr*$^{wt/tm1c}$ mice confirmed recombination of the conditional *Lifr* allele upon Cre exposure (WT = 389bp, KO = 172bp). Representative PCR analysis of testicular cDNA from (**C**) *Stra8-Cre*$^{-/-}$*; Lifr*$^{wt/tm1c}$ and *Stra8-Cre*$^{+/-}$*; Lifr*$^{wt/tm1c}$ and (**D**) *Amh-Cre*$^{-/-}$*; Lifr*$^{wt/tm1c}$ and *Amh-Cre*$^{+/-}$*; Lifr*$^{wt/tm1c}$ mice confirmed *Lifr* mRNA is expressed in germ cells and Sertoli cells, evidenced by the presence of a mutant transcript following Cre-mediated gDNA recombination (WT = 361bp, KO = 197bp). Western blot analysis of whole testis protein extracts from (**E**) *Stra8-Cre*$^{-/-}$*; Lifr*$^{tm1c/tm1c}$ (WT) and *Stra8-Cre*$^{+/-}$*; Lifr*$^{tm1c/tm1c}$ (GC-KO) and (**F**) *Amh-Cre*$^{-/-}$*; Lifr* $^{tm1c/tm1c}$ (WT) and Amh-*Cre*$^{+/-}$*; Lifr* $^{tm1c/tm1c}$ (SC-KO) mice. A significant reduction in testicular LIFR protein was observed only when *Lifr* was disrupted in Sertoli cells (unpaired *t*-test; p = 0.001). Tubulin-alpha (TUBA) was used as a loading control. Values are expressed as mean ± S.E.M of n = 7 samples per genotype.

4.2.2.2 Testicular Morphology is Normal in Germ Cell *Lifr*-KO Mice

Leukemia inhibitory factor receptor has been shown to be expressed in germ cells from the spermatogonial stage onwards (Jenab and Morris, 1998, Dorval-Coiffec *et al.*, 2005) suggesting that germ cell LIF/LIFR signalling may be required for the maintenance of normal spermatogenesis. However, testis weight did not differ between WT and GC-KO mice up to six months of age (Figure 4.4A) and no difference in testicular architecture was observed between WT and GC-KO mice (Figure 4.4B). In addition, morphologically mature spermatids were present in the cauda

epididymides of both WT and GC-KO mice (Figure 4.4C). These data demonstrate that LIFR is not essential in the germ cell population for normal spermatogenesis to occur.

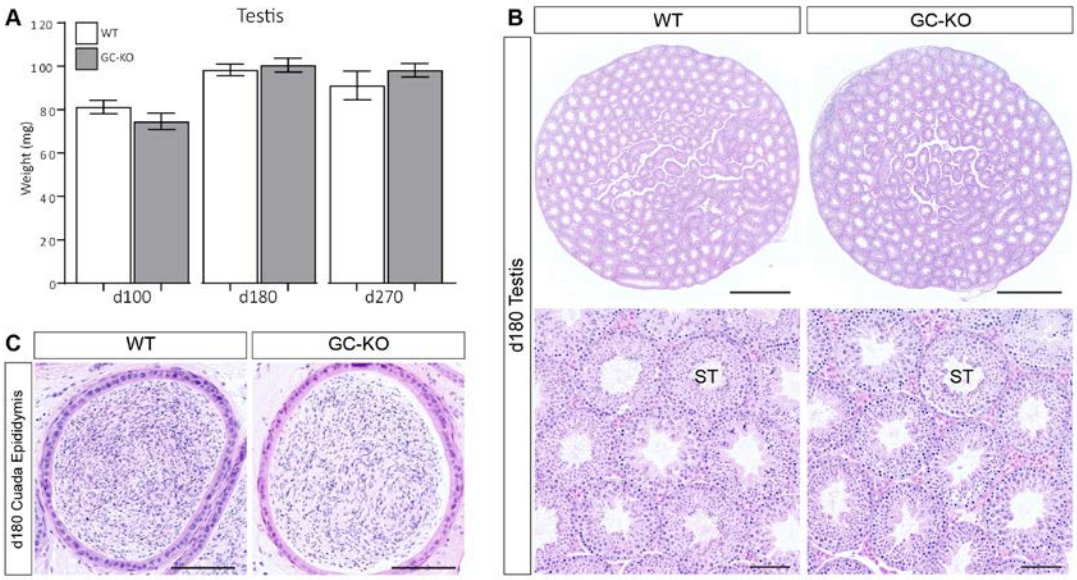


Figure 4.4 Normal Testicular Morphology in Germ Cell *Lifr*-KO Mice. (**A**) No difference in testis weight was observed between WT and GC-KO mice up to d270 (unpaired *t*-test). Values are expressed as mean ± S.E.M of n = ≥10 mice per age/genotype. (**B**) Representative H&E staining of WT and GC-KO testes at 6 months. No difference in testis morphology was noted between WT and GC-KO mice. ST = seminiferous tubule, scale = 1mm in top panels and 100µm in bottom panels. (**C**) Representative H&E analysis of the cauda epididymis confirmed the presence of morphologically mature spermatids in both WT and GC-KO mice. Scale = 100µm.

4.2.2.3 A Progressive Degenerative Testicular Phenotype is observed in Sertoli Cell *Lifr*-KO Mice

Having demonstrated that LIFR is dispensable in germ cells for normal spermatogenesis, the consequence of Sertoli cell LIFR ablation was next assessed. Initially, despite slight differences in testis weights (Figure 4.5), testicular histology was similar between WT and SC-KO animals up to d100 (Figure 4.6Ai-iii). However, degeneration of the seminiferous epithelium was observed from 6 months of age in SC-KO animals (Figure 4.6Aiv-v). This was accompanied first by an increase in testis weight at d180, followed by a reduction in testis weight at d270 (Figure 4.5). While seminiferous tubule degeneration/atrophy was widespread, there were still tubules within SC-KO testes that appeared normal (Figure 4.6Aiv-v). Additionally, morphologically mature spermatids were present in both WT and SC-KO cauda

epididymides at d180 (Figure 4.6B), suggesting that LIFR-deficient Sertoli cells can still support all steps of spermatogenesis.

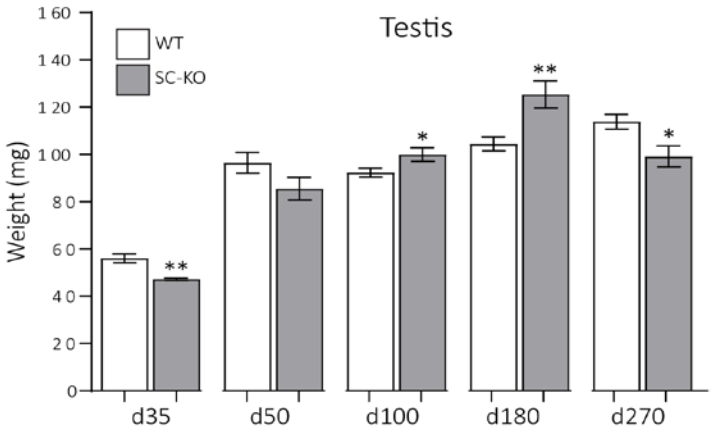


Figure 4.5 Testis Weight is Altered in Sertoli Cell *Lifr*-KO Mice. Significant differences in testis weights were noted between WT and SC-KO mice. At d35, a significant decrease in testis weight was observed in SC-KO animals (unpaired *t*-test; $p = 0.0028$. n = 7-9). At d50, testis weight was similar between WT and SC-KO animals (unpaired *t*-test; n = 9). At d100, a slight but significant increase in testis weight was observed in SC-KO mice (unpaired *t*-test; $p = 0.0354$; n = 13). This increase becomes more exaggerated at d180 (unpaired *t*-test; $p = 0.0028$; n = 12-16). By d270, a significant decrease in testis weight was observed in SC-KO mice (unpaired *t*-test; $p = 0.0195$; n = 9-11). Values are expressed mean ± S.E.M.

From d180, the seminiferous tubules in SC-KO mice appeared dilated with larger lumens than WT controls. This prompted a more in depth histological analysis of the SC-KO testis. Serial sectioning through entire SC-KO testes revealed regions of seminiferous tubule, often in close proximity to the rete testis, with increasing concentrations of sloughed germ cells/cellular debris and signs of apparent sperm stasis (Figure 4.6C). This is consistent with increased back-pressure into the testis, similar to that observed in several other rodent models (Jeffs *et al.*, 2001, O'Hara *et al.*, 2011, Heuser *et al.*, 2013, Hess, 2014). To determine whether there is an additional effect of LIFR ablation from both germ cells and Sertoli cells together, double knockout mice were generated. However, testis histology in double mutants was similar to that of the SC-KO animals, further confirming that LIFR is not required in germ cells (Figure 4.7). Interestingly, in contrast to the single SC-KO mutants described above, there is no difference in testis weight between WT and SC+GC double mutants or their GC-KO and SC-KO littermate controls at d180 (Figure 4.7).

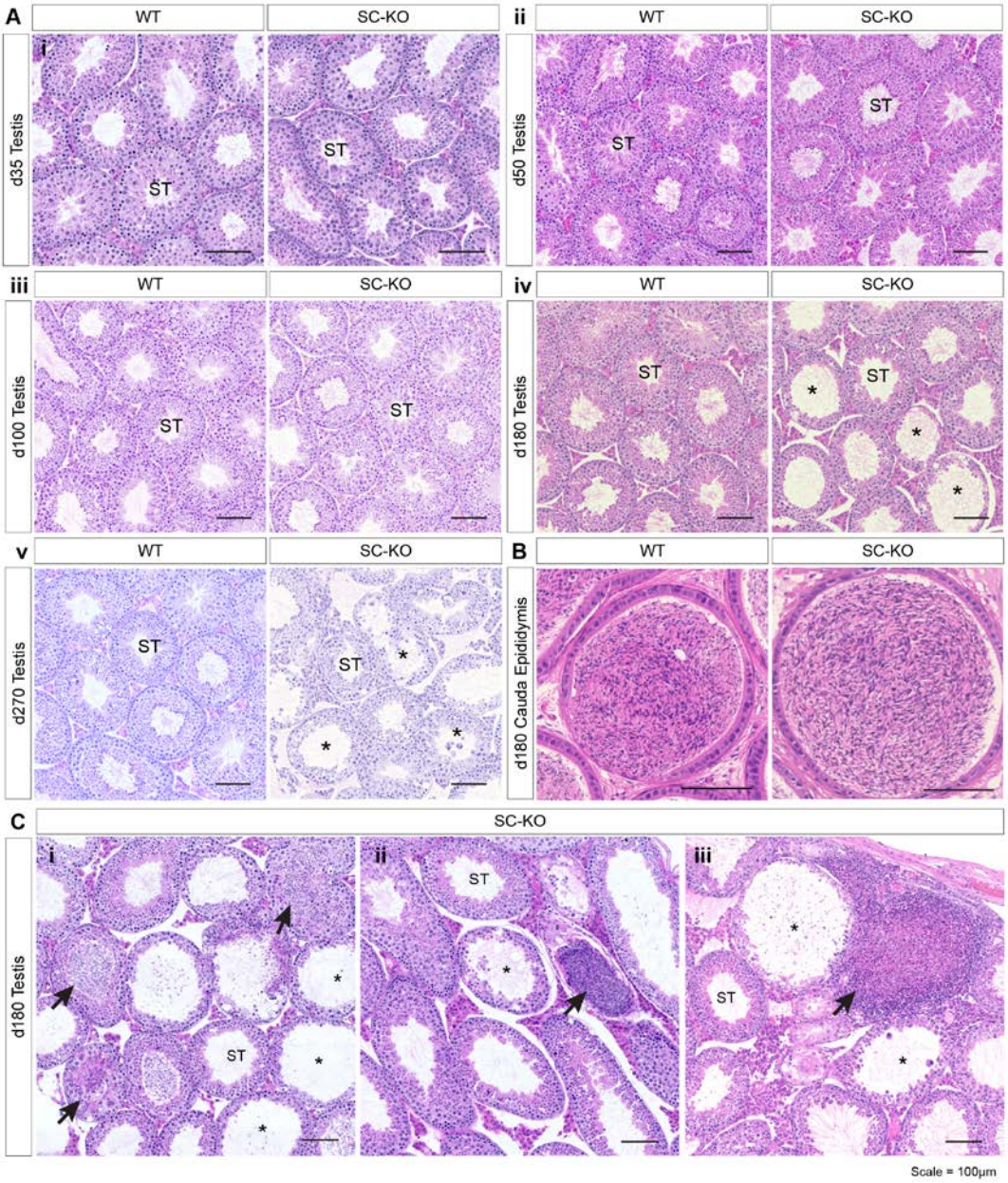


Figure 4.6 Disrupted Testicular Histology in Sertoli Cell *Lifr*-KO Mice. Representative H&E staining of WT and SC-KO testes up to d270. (**A**) Testis histology was normal in SC-KO mice up to d100 (i-iii). At d180, degeneration of the seminiferous epithelium was observed in SC-KO testes (iv). While morphologically normal tubules (ST) were observed in the SC-KO testis, a large proportion of tubules appear distended and vacuolation of the seminiferous epithelium was noted (asterisks). (**B**) Morphologically mature spermatids were present in cauda epidiymides of both WT and SC-KO mice at d180. (**C**) Serial sectioning through entire SC-KO testes revealed sections of tubule with increasing concentrations of sloughed germ cells/cellular debris and signs of apparent sperm stasis (arrows). Scale bars = 100µm.

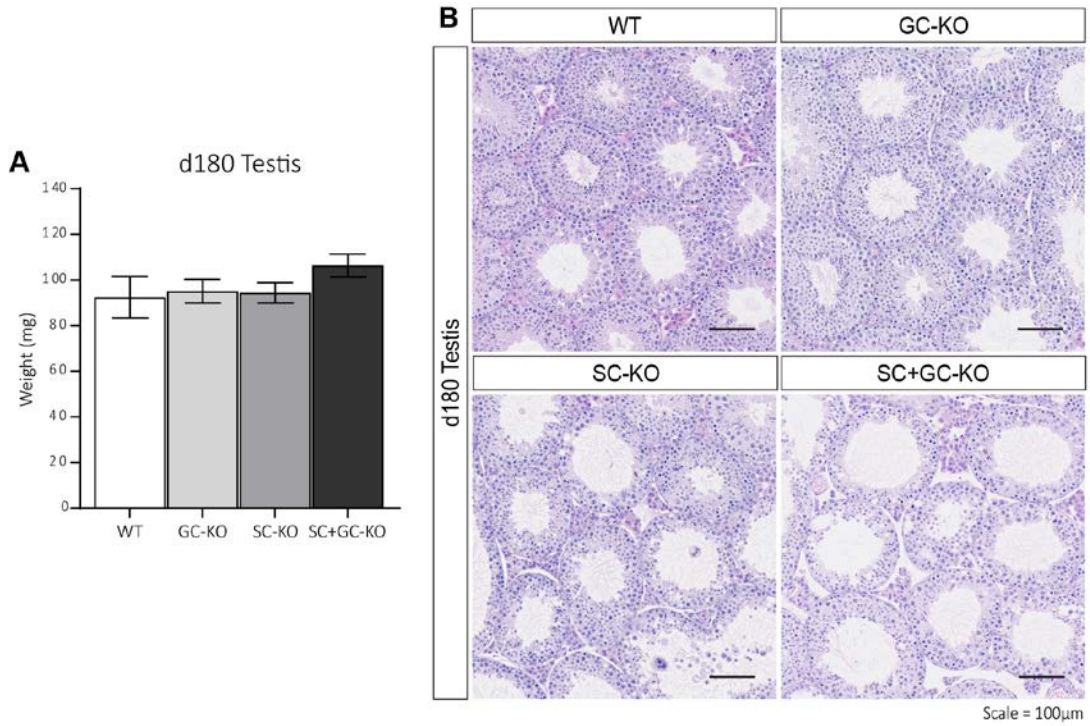


Figure 4.7 No additional Effect is observed when *Lifr* is Disrupted Both in Germ Cells and Sertoli Cells. (**A**) No difference in testis weight d180 was observed when *Lifr* is disrupted both in germ cells and in Sertoli cells (SC+GC-KO; 1-way AVOVA, n = 6-13). (**B**) Representative H&E staining of WT, GC-KO, SC-KO and SC+GC-KO testes at d180. Testicular phenotype was similar in both SC-KO and GC+SC-KO confirming that *Lifr* is dispensable in germ cells but not Sertoli cells for normal spermatogenic function. Scale = 100µm.

4.2.2.4 Sertoli Cell and Spermatogonia Volume is Reduced in d180 Sertoli cell *Lifr*-KO Mice

To determine whether sloughing of a specific germ cell population was responsible for the tubule obstruction observed in SC-KO testes, the expression of germ cell-specific 'biomarker' mRNAs was measured. No difference in the expression of *Stra8*, *Spo11* or *Tpn1* was detected between WT and SC-KO testes at d180 (Figure 4.8A), suggesting no major deficit in the spermatogonia, spermatocyte or spermatid populations respectively. However, stereological analysis of the seminiferous epithelium revealed a reduction in the volume of Sertoli cells and spermatogonia per testis (Figure 4.8B). Despite this reduction, no differences in spermatocyte and spermatid volume was noted (Figure 4.8B). Next, immunostaining for activated caspase 3 (CASP3), a marker of apoptosis, was conducted to inform whether increased apoptosis may explain the observed reduction in Sertoli cells and spermatogonia.

When quantified, no difference in the proportion of activated CASP3-positive tubules per section, or CASP3-positive cells in the basal region of seminiferous tubules were noted between WT and SC-KO animals (Figure 4.8C). However, CASP3 immunoreactivity appeared more widespread throughout the seminiferous epithelium in SC-KO testes. In WT testes, apoptotic cells were generally confined to the basal regions of the seminiferous epithelium. Finally, to determine whether a perturbation in the spermatogonial stem cell (SSC) niche may be responsible for the reduction in spermatogonia, mRNA expression of Sertoli-cell derived factors which have been shown to be important for proper maintenance of SSCs (Garcia *et al.*, 2014) were measured. Expression of *Gdnf, Cy26b1* and *Kitl* was similar in WT and SC-KO testes (Figure 4.8D), suggesting that dysregulation of the SSC niche may not explain the reduction in spermatogonia in the SC-KO testis.

Figure 4.8 Sertoli cell and Spermatogonia Volume is Reduced in Sertoli Cell *Lifr*-KO Testes. *Figure on following page* (**A**) Expression of spermatogonia (*Stra*8), spermatocyte (*Spo11*) and spermatid (*Tpn1*) specific transcripts. mRNA expression was similar between WT and SC-KO testes at d180 of age (unpaired *t*-test, n = 9-10). (**B**) Stereological analysis revealed a reduction in Sertoli cell and spermatogonia absolute nuclear volume in the SC-KO testes at d180 (unpaired *t*-test; p = 0.0172 and 0.0325 respectively; n = 7). (**C**) Representative immunostaining for activated CASP3 (brown), as a marker of apoptosis, in WT and SC-KO testes at d180. No difference in the number of CASP3-positive tubules, or CASP3-positive cells lining the basal region of the tubules (black arrows, higher magnification insets) was noted between WT and SC-KO testes (unpaired *t*-test; n = 5-6). CASP3 immunoreactivity was occasionally observed throughout the seminiferous epithelium (open arrowheads) in SC-KO animals. Primary antibody negative control and induced Sertoli cell death positive controls are included. Scale bars = 100µm. (**D**) Expression of *Gdnf*, *Cy26b1* and *Kitl* was similar in WT and SC-KO testes. All values are expressed as the mean ± S.E.M.

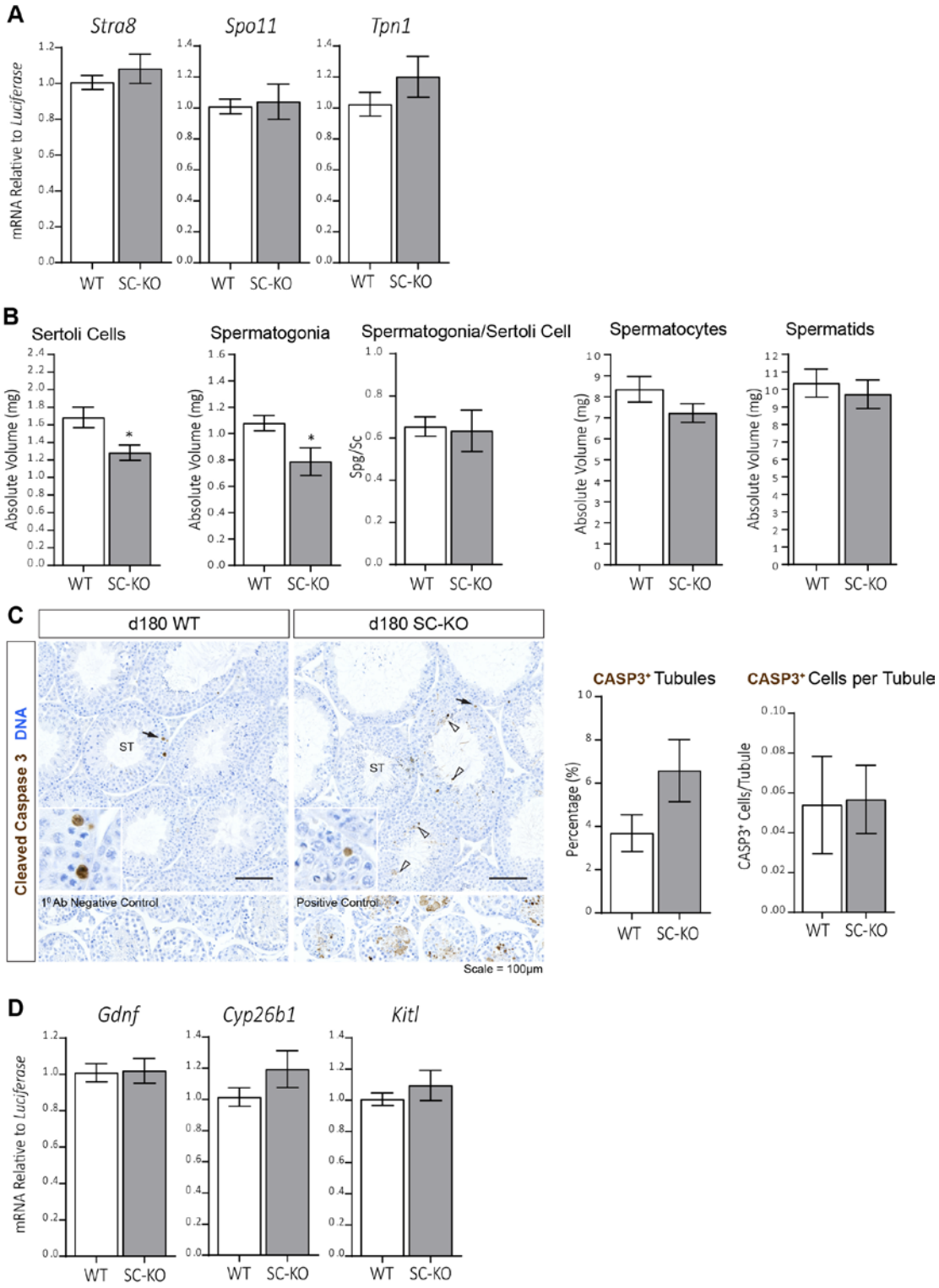


Figure 4.8 Sertoli cell and Spermatogonia Volume is Reduced in Sertoli Cell *Lifr*-KO Testes. *Legend on preceding page*

4.2.2.5 Blood-Testis-Barrier Integrity is maintained in Sertoli Cell *Lifr*-KO Testes

The blood-testis-barrier (BTB) plays an important role in maintaining the appropriate intratubular environment required for normal spermatogenesis. Previous reports have suggested a role for IL-6 family cytokines, of which LIF is a member, in the regulation of BTB function (Perez *et al.*, 2012, Zhang *et al.*, 2014). Therefore, the ability of LIFR-deficient Sertoli cells to maintain the integrity of the BTB was determined. Functional assessment of the BTB using a biotin tracer confirmed the BTB remained intact in the SC-KO testis (Figure 4.9A). This was consistent with normal mRNA expression of the BTB associated genes *Ocdn*, *Cldn3* or *Cld11* (Figure 4.9B).

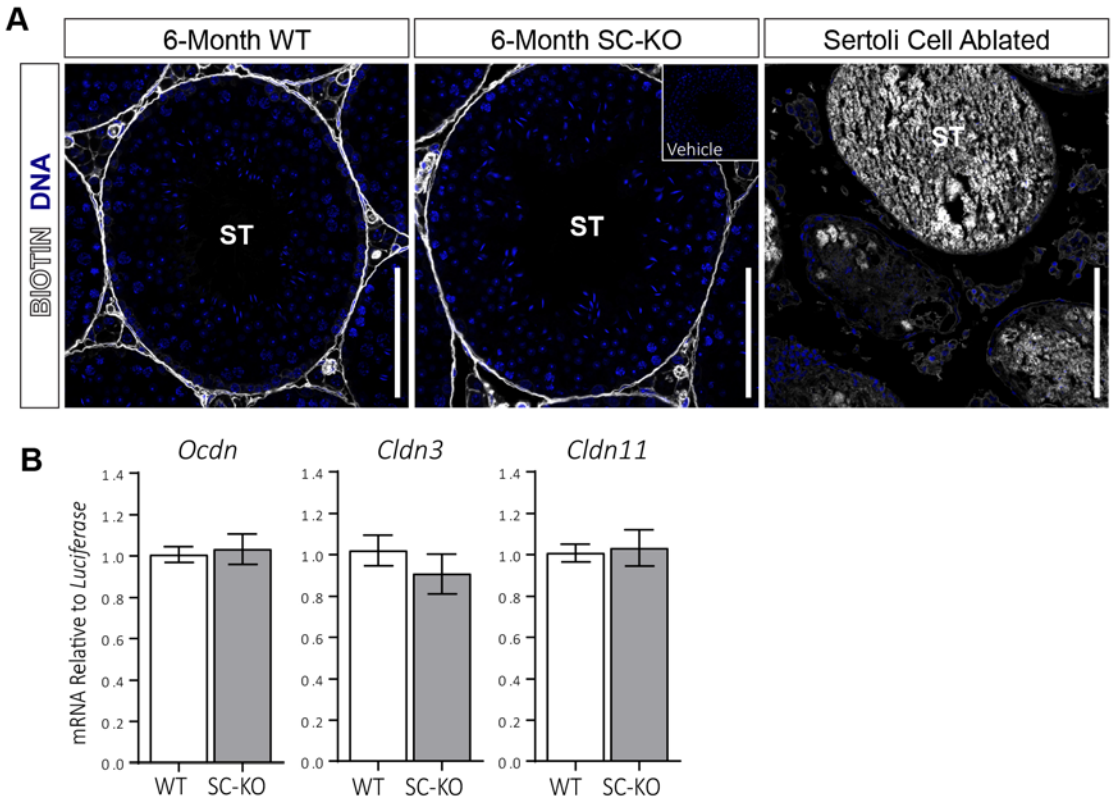


Figure 4.9 Analysis of Blood-Testis-Barrier Integrity in Sertoli Cell *Lifr*-KO Testes. (**A**) A biotin tracer injected into the testis interstitium was restricted to the interstitial compartment between seminiferous tubules (ST) confirming the blood-testis-barrier remained intact in SC-KO mice at d180. A Sertoli cell-ablated testis was included as a positive control demonstrating biotin infiltration into seminiferous tubules. Scale bars = 100µm. (**B**) Expression of the blood-testis-barrier associated mRNAs *Ocdn*, *Cldn3* or *Cld11* was unaltered in SC-KO testes at 6 months (unpaired *t*-test; n = 9-10). Values are expressed as mean ± S.E.M.

4.3 Discussion

Development and function of the mammalian testis is subject to endocrine regulation by the HPG axis, as well as a complex suite of paracrine factors, which ensure the adequate production of both sperm and androgens in adult males. The experiments conducted within this chapter were designed with the aim of identifying the contribution of leukemia inhibitory factor receptor (LIFR) signalling to testis development and function. Utilising a series of mutant *Lifr* mouse models, the data presented herein demonstrate that LIFR does not appear to be crucial for prenatal testicular development. Conversely, LIFR in Sertoli cells, but not in germ cells, is required for the maintenance of normal structure/function of the seminiferous tubules in adulthood. At the present time these data are believed to provide the first *in vivo* evidence demonstrating the importance of LIFR signalling in Sertoli cells.

Both *Lif* and *Lifr*, are expressed in the prenatal rodent testis from embryonic day (e) 12.5 (Piquet-Pellorce *et al.*, 2000, Molyneaux *et al.*, 2003, Dorval-Coiffec *et al.*, 2005) suggesting a role in testis organogenesis. In the present studies, normal testicular architecture, characterised by fully formed seminiferous cords containing Sertoli and germ cells, and abundant interstitial fetal Leydig cells, was observed in LIFR-deficient mice on the day of birth, suggesting that LIFR is dispensable for the establishment of a morphologically normal testis. However, due to time constraints, analysis of constitutive LIFR-KO testis was limited to a morphological assessment of testis structure, thus further stereological analyses would be required to determine whether loss of LIFR results in altered numbers of germ cells, Sertoli cells and Leydig cells. Furthermore, LIFR-KO testes were analysed on post-natal day 0. As such the possibility that LIFR-loss affects testicular organogenesis at earlier developmental time points, which may not be apparent at d0 cannot be ruled out.

It has been reported previously that the number and morphology of primordial germ cells (PGC) is normal in LIFR-deficient mice when analysed at e10.5-13.5 (Ware *et al.*, 1995). Conversely, a slight reduction in PCG number was noted in gp130-deficient male fetuses (gp130 is the signal transducer with which LIFR must dimerise with to induce signalling) (Molyneaux *et al.*, 2003). Whilst neither study made comment upon the developing somatic components of the fetal mouse testis, a recent study which

aimed to identify genes involved in congenital abnormalities of the kidney and urogenital tract (CAKUT), noted a form of cryptorchidism in a CAKUT patient heterozygous for a hypomorphic *LIFR* mutation (Kosfeld *et al.*, 2017). The authors go on to suggest that disruption of murine *Lifr* also results in a potential form of cryptorchidism, characterised by an abnormal ligament attaching the testis to the dorsal aorta, present at e18.5. Such a defect was not noted in the present study, possibly owing to the genetic background of the *Lifr* mutants – in the above study animals were maintained on an outbred Ztm:NMRI background which may be more susceptible to development of urogenital defects. Alternatively, regression of this structure may occur between e18.5 and d0 – the time point at which constitutive LIFR-KO animals were studied herein. It may also be possible that such a defect went unnoticed during the course of the present studies. Nevertheless, it can be concluded from the data presented in this chapter that LIFR is not required for the development of normal prenatal testicular structure.

Constitutive *Lifr* deletion results in a lethal phenotype prior to weaning, thus LIFR deficient mice cannot be used for the study of adult testis development/function. Therefore a Cre/*loxP* approach to disrupt *Lifr* in germ cells and Sertoli cells was used. Cre recombinase is reported to be active in spermatogonia from postnatal day 3 in the *TgStra8-icre*^{1Reb} mouse line (Sadate-Ngatchou *et al.*, 2008) which was used to disrupt *Lifr* in germ cells. In the rat testis, spermatogonia have been suggested to be a major target of LIFR signalling (Dorval-Coiffec *et al.*, 2005), although this conclusion was arrived at through the comparison of d9 spermatogonia to d20 somatic cells, thus may not accurately represent expression in the adult testis. Surprisingly, analysis of GC-KO mice suggested that LIFR is in fact dispensable in germ cells, from the spermatogonial stage onward, for normal spermatogenesis in mice. Functional redundancy between LIF and other IL6-family members signalling through gp130 provide a possible explanation, however, a previous report using *Tnap-cre* to delete gp130 from primordial germ cells reported no phenotype in mutant male mice (Molyneaux *et al.*, 2003). No mention of the endpoints measured, nor the age of the animals was made in that study, thus the development of a spermatogenic defect in ageing gp130-deficient animals cannot be ruled out.

A number of studies have documented wide-spread expression of *Lif* and *Lifr* in both germ and somatic cell populations in the adult rodent testis (Jenab and Morris, 1998, Piquet-Pellorce *et al.*, 2000, Molyneaux *et al.*, 2003, Dorval-Coiffec *et al.*, 2005), implicating LIF/LIFR signalling as a potential regulator of testicular function. Indeed, *in vitro* experiments have demonstrated that LIF can enhance the survival of gonocytes and Sertoli cells in a primary co-culture system (De Miguel *et al.*, 1996) and that LIF stimulates spermatogonial proliferation and/or survival when added to primary cultures of seminiferous tubule segments (Dorval-Coiffec *et al.*, 2005). However, Jenab and Morris (1998) reported that LIF/LIFR signalling is active in rat Sertoli cells, but possibly not in germ cells, albeit *in vitro*, indicating that the proliferative and/or anti-apoptotic effects of LIF on gonocytes/spermatogonia are likely mediated by the Sertoli cells present in the cultures. Indeed, the studies presented herein demonstrate that LIFR is dispensable in germ cells for normal spermatogenesis to occur, but Sertoli cell LIFR ablation appears to cause spermatogenic defects.

When LIFR was deleted from the Sertoli cell population, postnatal testis development appeared normal, based on histological examination of WT and SC-KO testes up to d100. Initially, SC-KO testes appeared slightly lighter than those of WT controls but the basis for this is unclear at the present time. Unfortunately time constraints of the current project did not permit a more detailed stereological analysis of Sertoli and/or germ cells in the developing pubertal testis which may inform on these subtle weight discrepancies. Whilst loss of Sertoli cell LIFR did not appear to have a major impact on postnatal testicular development, analysis of SC-KO testes at d180 and d270 showed that LIFR is required to support normal spermatogenesis. Despite an apparent reduction in Sertoli cells and spermatogonia, testis weight is significantly increased in 6 month old SC-KO mice and a large proportion of tubules appeared dilated, likely due to mechanical testicular blockage/sperm stasis. However, the molecular mechanism(s) behind disruption to the seminiferous epithelium remains to be elucidated. One function of mature Sertoli cells is the production of seminiferous tubule fluid (Jegou *et al.*, 1982) which is required for the appropriate development of sperm as well as their transport out of the testis. As such, defective fluid production by LIFR-deficient Sertoli cells may explain the observed sperm stasis in SC-KO testes.

In addition to Sertoli cells, LIFR is also expressed by Leydig cells (Jenab and Morris, 1998, Dorval-Coiffec *et al.*, 2005), as well as their putative stem/progenitors (Ge *et al.*, 2006, Jiang *et al.*, 2014), suggesting LIF/LIFR signalling may play a role in regulating Leydig cell development and steroidogenic function. Indeed both LIF and the closely related cytokine oncostatin M (OSM), which can also signal *via* LIFR have been implicated in development of the adult Leydig cell population (Teerds *et al.*, 2007b, O'Shaughnessy *et al.*, 2008). Furthermore, *in vitro* studies have demonstrated LIF can have both inhibitory and stimulatory effects on Leydig cell steroidogenesis (Mauduit *et al.*, 2001, Wang *et al.*, 2016b). However, a definitive role for LIFR signalling in Leydig cell development and steroidogenic function is yet to be established, but will certainly be an interesting avenue of further study.

To conclude, the studies presented in this chapter provide the first *in vivo* evidence that LIFR signalling is required for normal testicular function in adulthood. Specifically, a requirement for Sertoli cell LIFR in the maintenance of normal spermatogenic function has been identified, widening our understanding of the paracrine network in the testis. Elucidation of the precise mechanism(s) by which LIFR signalling contributes to Sertoli cell function will further improve our understanding of this fundamentally important cell.

5 The Effect of Human Adipose-Derived Perivascular Stem Cells in a Rat Model of Leydig Cell Injury

5.1 Introduction

Establishment and maintenance of the male phenotype requires adequate production and action of androgens (e.g. testosterone). Further to their well-established role in male reproductive function, increasing evidence suggest androgens also support overall male general health. Low circulating testosterone levels have been linked to an increased risk of developing cardiometabolic disorders, which are also often associated with advancing age (Kupelian *et al.*, 2006, Farrell *et al.*, 2008, Kupelian *et al.*, 2008, Brand *et al.*, 2014, Pye *et al.*, 2014). Indeed, circulating testosterone levels decline as males age (Morley *et al.*, 1997, Harman *et al.*, 2001, Feldman *et al.*, 2002, Wu *et al.*, 2008, Fabbri *et al.*, 2016), often due to diminished ability of testicular Leydig cells to produce androgens, resulting in a state of primary hypogonadism. Androgen replacement therapy is widely used to treat hypogonadism although the risks and benefits associated with the administration of exogenous testosterone are not entirely clear (Bassil *et al.*, 2009). Recent efforts have thus been made to develop novel therapies that promote and/or maintain endogenous testicular testosterone production within a normal physiological range.

In recent years, considerable attention has been focussed on the development of stem cell-based regenerative therapies to treat a number of diseases (Trounson and McDonald, 2015). A wide variety of stem cell types, from pluripotent embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, to multipotent mesenchymal stem cells (MSC), continue to be investigated for their potential utility in the field of regenerative medicine. For example, human ES cells differentiated into retinal pigment epithelial cells *in vitro*, are reported to have a positive impact on vision in animal models (Lu *et al.*, 2009) and when transplanted in patients with age-related macular degeneration or Stargardt's macular dystrophy (Schwartz *et al.*, 2015, Song *et al.*, 2015), although the immune privilege of the eye may be a significant factor in these transplantation successes. Additionally, ethical controversies surrounding the use of ES cells, as well as safety concerns, including tumorigenicity (Amariglio *et al.*,

2009), associated with the use of pluripotent stem cells types may impede their translation into clinical practice. As such, multipotent MSCs represent an attractive alternative for regenerative therapies. MSCs can be isolated from multiple tissues (Crisan *et al.*, 2008) and may act as either progenitor cells, differentiating into various cell lineages to effect tissue repair; or play a paracrine role by secreting factors which promote endogenous tissue regeneration (Chen *et al.*, 2009a, Chen *et al.*, 2013).

A number of *in vitro* and *in vivo* studies have described the generation of Leydig-like cells from stem cell populations isolated from rodent testes (Ge *et al.*, 2006, Zhang *et al.*, 2013, Jiang *et al.*, 2014, Odeh *et al.*, 2014, Li *et al.*, 2016, Zang *et al.*, 2017). While these studies have increased our understanding of stem Leydig cells and their differentiation, extraction of stem cells from a patient's testis would be impractical as a therapeutic approach. Adipose tissue is a potentially promising source of stem cells as it is easily accessible with minimally invasive techniques. Murine adipose-derived stem cells have been driven down a steroidogenic lineage *in vitro* after transfection with steroidogenic factor-1 (Gondo *et al.*, 2008), although these cells appeared to favour the production of glucocorticoids over androgens. Additionally, in an experimentally induced ageing model, rat adipose derived stem cells were reported to have a beneficial effect on testicular function *in vivo* (Yang *et al.*, 2015a). However, to date, the ability of human adipose-derived perivascular stem cells (hAd-PSC) to promote Leydig cell function has not been assessed.

5.1.1 Hypothesis & Aims

The hypothesis underlying this chapter is that human adipose-derived perivascular stem cells (hAd-PSC) could be used for the treatment of Leydig cell dysfunction. As such, the overall aim of the experiments herein was to assess the regenerative capacity of hAd-PSCs to promote endogenous testosterone production as a potential therapeutic strategy for the treatment of hypogonadism. Firstly, the ability of hAd-PSCs to form Leydig-like cells *in vitro* was determined. A well-established rat model of Leydig cell ablation-regeneration was then utilised to assess the effect of hAd-PSCs on Leydig cell regeneration *in vivo*. Following ethane dimethanesulphonate (EDS)-mediated Leydig cell ablation, hAd-PSCs were transplanted into the rat testis interstitium and Leydig cell regeneration was monitored *via* serial measurements of circulating luteinising

hormone (LH) and testosterone (T). The experimental overview is outlined in Figure 5.1.

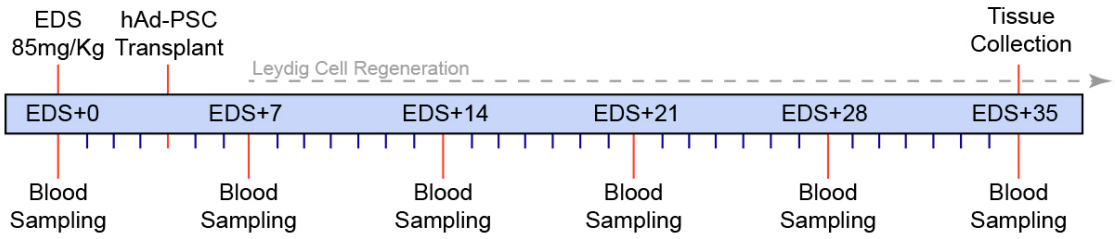


Figure 5.1 Experimental Overview. Leydig cells were ablated using a single dose of ethane dimethanesulphonate (EDS). Human adipose-derived perivascular stem cells (hAd-PSCs) were transplanted into Leydig cell ablated testes 4 days after EDS administration. Tail-vein blood samples were collected weekly for the measurement of circulating testosterone and luteinising hormone. Animals were sacrificed after 5 weeks (EDS+35).

5.2 Results

5.2.1 Effect of Differentiation Inducing Medium on hAd-PSCs *In Vitro*

To test whether hAd-PSCs could form testosterone-producing cells *in vitro*, hAd-PSCs (see section 2.7.1) were exposed to a differentiation inducing medium (DIM; (Ge *et al.*, 2006, Jiang *et al.*, 2014)) as described in section 2.7.1.1. After one week, cells were harvested and the mRNA expression of genes involved in androgen biosynthesis was measured by qRT-PCR and compared to control cells cultured in expansion media (EM; see section 2.7.1). Exposure of hAd-PSCs to DIM induced the expression of *STAR* and *CYP11A1* mRNA however, neither *CYP17A1* nor *HSD17B3* were detectable (Figure 5.2). To assess the utility of antibodies raised against human nuclear antigen (HuNu) and human leukocyte antigen class 1 (HLA1) for the identification of transplanted cells in rat testes, fluorescent immunocytochemistry was performed (see section 2.2.4). Expression of both markers was observed in EM and DIM cultured hAd-PSCs (Figure 5.3).

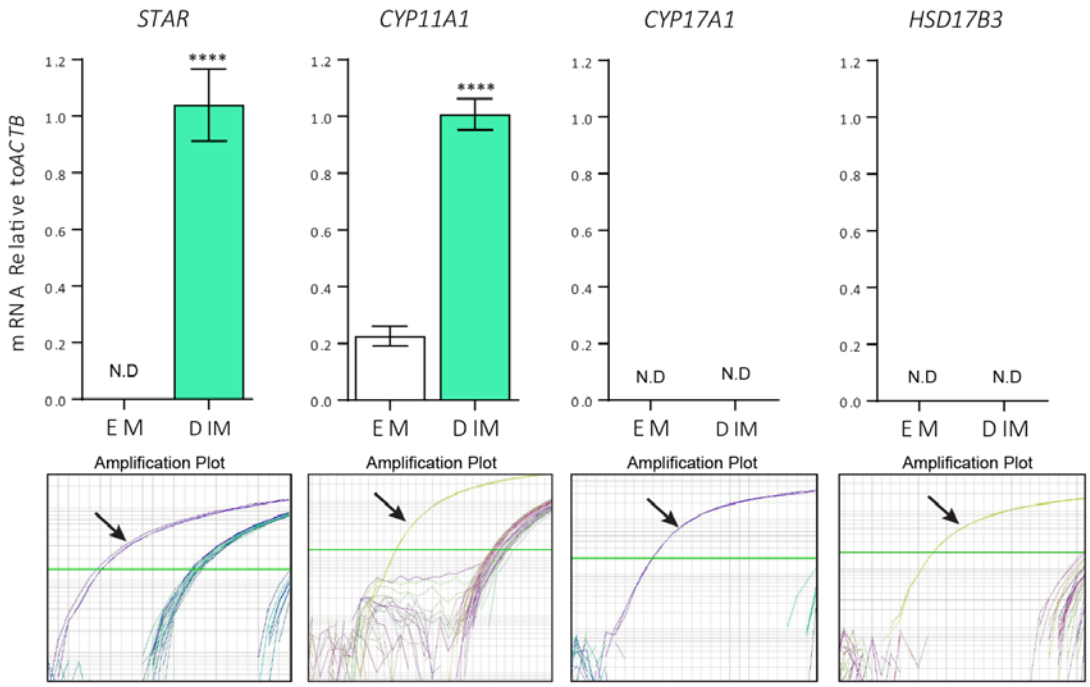


Figure 5.2 mRNA Expression of Leydig Cell Steroidogenic Enzymes in DIM-Exposed hAD-PSCs. Expression of *STAR* (steroidogenic acute regulatory protein) and *CYP11A1* (P450 cholesterol side-chain cleavage enzyme) was induced in hAd-PSCs after one week culture in differentiation inducing media (DIM; *t*-test; p = <0.0001). Neither *CYP17A1* (17α-hydroxylase, 17,20-lyase) nor *HSD17B3* (hydroxysteroid dehydrogenase 17-beta type 3) were detectable. Arrows in bottom panels indicate the amplification of adult human testis cDNA as a positive control. Values represent the mean ± SEM from n=6 separate wells per group. N.D = Not detected. The experiment was repeated on two separate occasions with similar results.

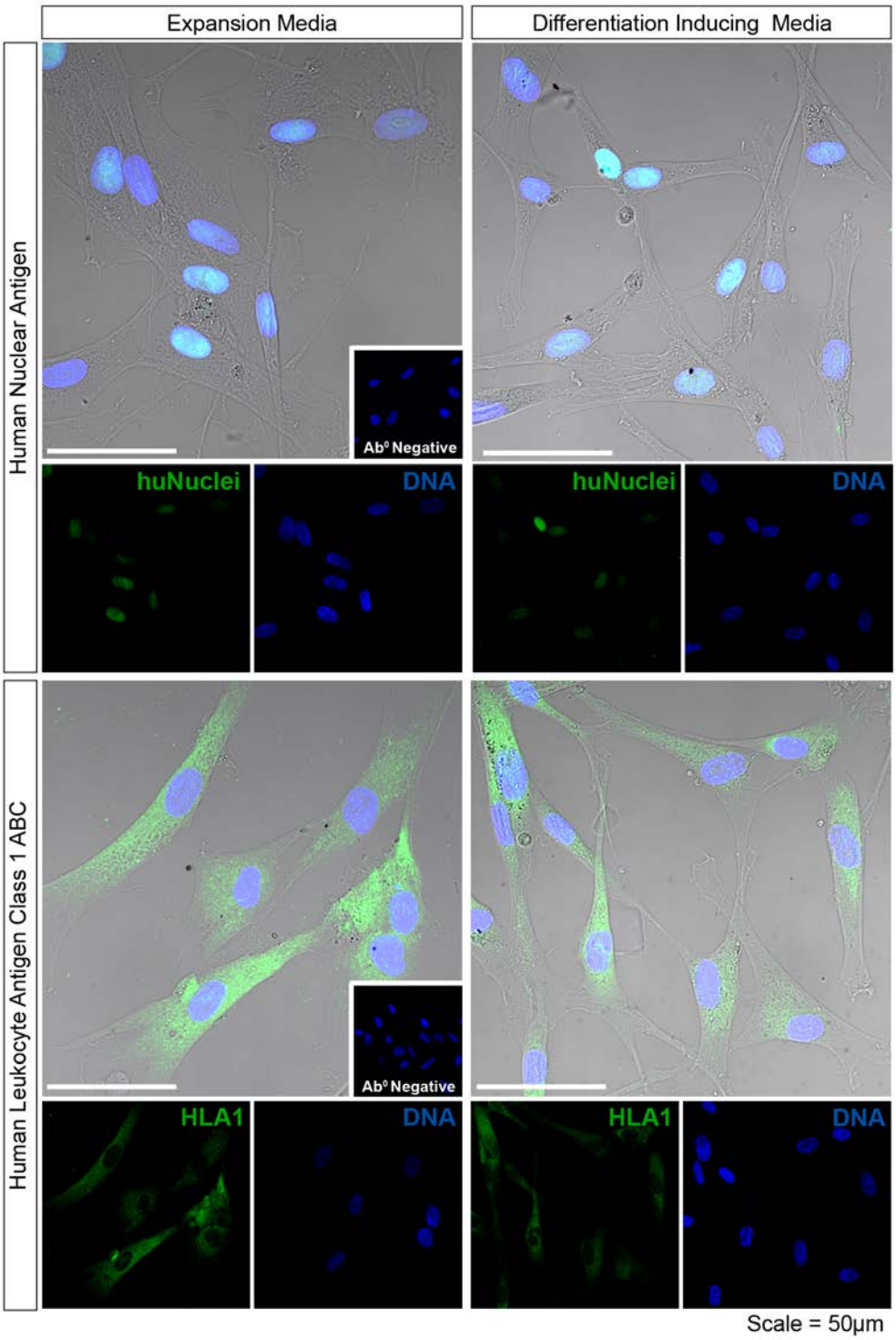


Figure 5.3 Validation of Antibodies Raised against Human-specific Proteins in Cultured hAd-PSCs. Both human nuclear antigen (huNuclei; top panels) and human leukocyte antigen class 1 (HLA1; bottom panels) were detected in human adipose-derived perivascular stem cells (hAd-PSC) exposed to either expansion media (left) or differentiation inducing media (right) media. In each case the cells have a spindle-shaped, fibroblast-like morphology. Insets = primary antibody negative controls. Scale = 50µm.

5.2.2 Effect of hAd-PSC on Leydig Cell Regeneration *In Vivo*

5.2.2.1 Recovery of Circulating Testosterone

In the rat testis, complete ablation of Leydig cells occurs within 3-7 days following EDS administration, followed by regeneration of the Leydig cell population and restoration of circulating testosterone levels from 3-7 weeks (Morris and McCluckie, 1979, Kerr *et al.*, 1985, O'Shaughnessy *et al.*, 2008, Kilcoyne *et al.*, 2014). To determine the effect of hAd-PSCs on Leydig cell regeneration, cells were cultured in EM or DIM, and transplanted into the rat testis interstitium 4 days after EDS treatment (see section 2.1.4.3). Control groups included (i) Vehicle+Sham – no EDS was administered, animals underwent surgery to expose testes but no cells were transplanted. (ii) EDS+Sham – Animals were treated with EDS to ablate Leydig cells and underwent surgery to expose testes but no cells were transplanted. Leydig cell regeneration was monitored *via* serial measurements of circulating testosterone and luteinising hormone. In line with previous studies (Kerr *et al.*, 1985, O'Shaughnessy *et al.*, 2008), testosterone was undetectable 7 days after EDS treatment but returned to control levels by 21 days (Figure 5.4Ai and ii), confirming successful Leydig cell ablation and regeneration. To identify any effect of the transplanted hAd-PSCs on recovery of circulating testosterone between these time points, area under the curve (AUC) was calculated for each group. As expected, AUC was significantly decreased in all EDS treated groups compared to Vehicle+Sham controls. However, no difference was observed when EM or DIM cultured hAd-PSCs were transplanted into the testis (Figure 5.4Aiii). In line with the circulating testosterone profile (Figure 5.4A), an increase in LH, peaking at 14 days, was observed following EDS treatment (Figure 5.4Bi and ii). AUC of the LH surge was similar between EDS+Sham and both hAd-PSC transplanted groups. However, the AUC was significantly higher in the EDS+EM group compared to the EDS+DIM group (Figure 5.4Biii).

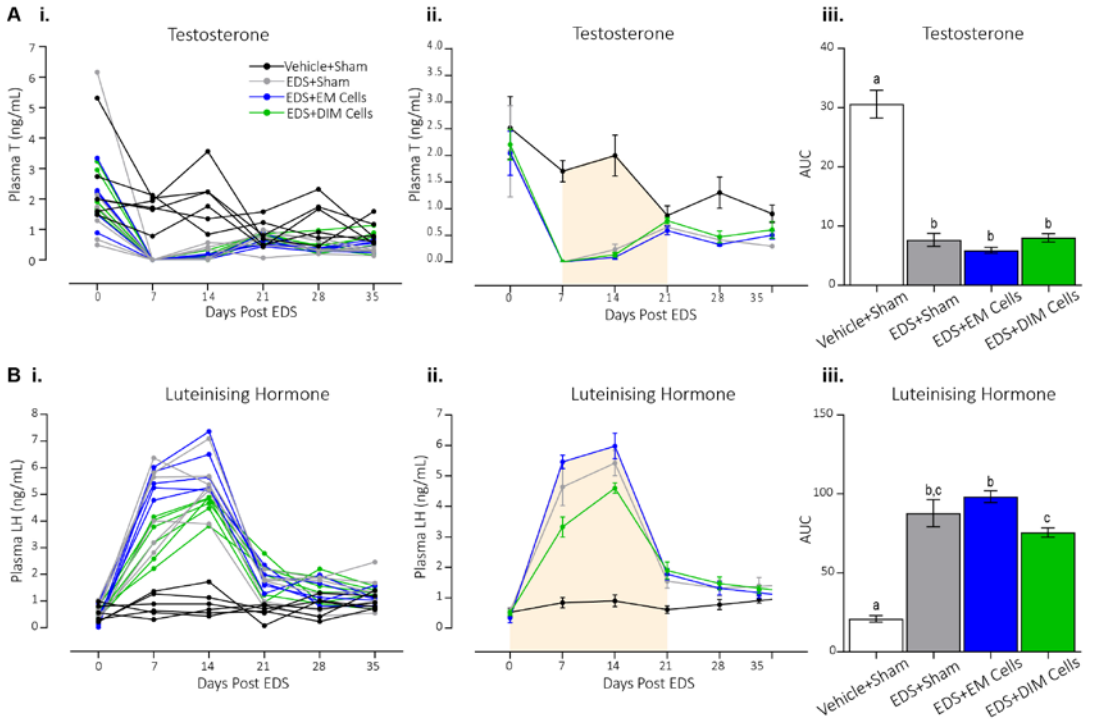


Figure 5.4 Circulating Hormones during Leydig Cell Regeneration. Circulating testosterone (**A**) and luteinising hormone (**B**) were measured in sera from tail vein blood samples collected weekly throughout the experiment (5 weeks). In each case, individual animals are plotted in (**i**) and the mean ±SEM are plotted in (**ii**). The orange shaded areas highlight the proportion of the data used for area under the curve (AUC) analyses shown in (**iii**), based on the time taken for testosterone to return to control levels (EDS+21). 1-way ANOVAs were used to identify significant differences between experimental groups (p = <0.0001 for both testosterone and LH). Tukey's post-hoc analysis was used to compare means between groups where a shared letter denotes no significant difference. n = 5-6 animals per group.

5.2.2.2 Recovery of Testis and Seminal Vesicle Weight

When animals were sacrificed 35 days post EDS, no difference in body weight was observed between groups, suggesting neither EDS nor hAd-PSCs had major negative side-effects (Figure 5.5A). Conversely, significant differences in testis and seminal vesicle weights were noted (Figure 5.5B and C respectively). Recovery of testis weight to that of Vehicle+Sham controls was observed in the EDS+Sham and EDS+DIM groups whereas EDS+EM testes remained significantly smaller at 35 days post EDS. In the case of seminal vesicle weight, recovery equivalent to Vehicle+Sham was only observed in the EDS+DIM group. To determine whether hAd-PSCs remained within the testis 1 month post-transplantation, genomic DNA was isolated from frozen testes and interrogated for the presence of human-specific DNA (*HUMSAT17A*). However,

no human DNA was detectable in testes which had been transplanted with either EM or DIM hAd-PSCs (Figure 5.5D).

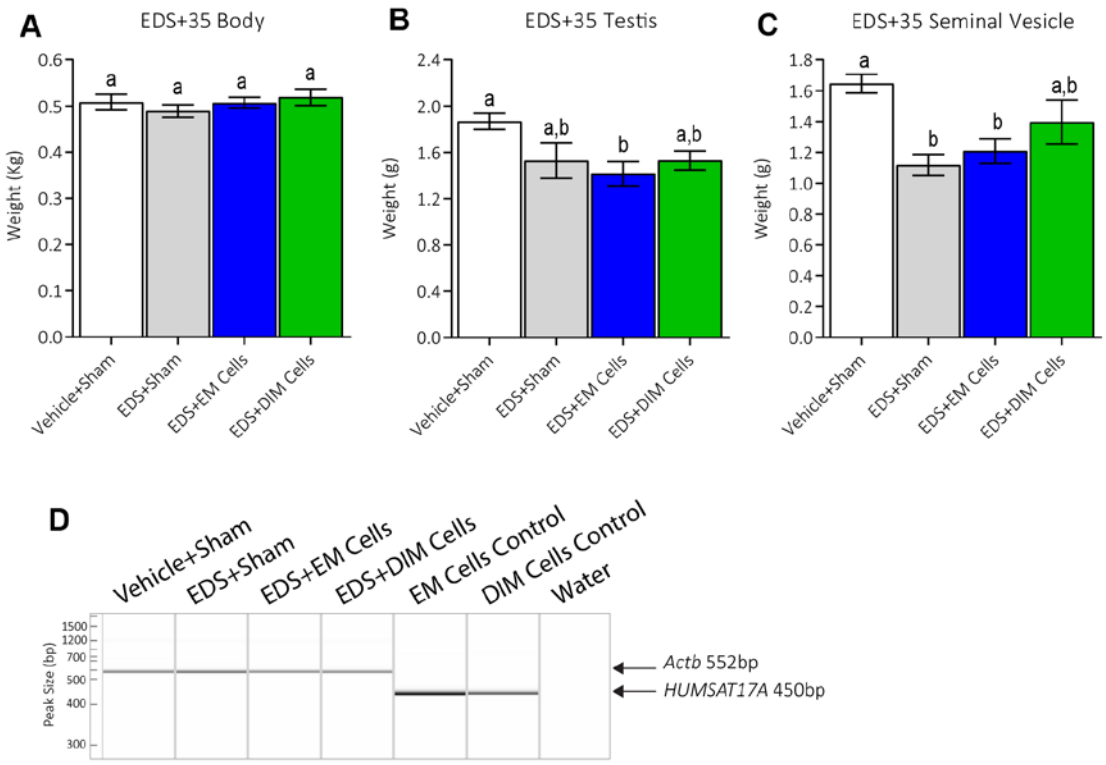


Figure 5.5 Recovery of Testis and Seminal Vesicle Weight. (A) No difference in body weight was noted between experimental groups at the end of the experiment (EDS+35; 1-way ANOVA). (B) Testis weight recovered in EDS+Sham and EDS+DIM groups but remained significantly lower in the EDS+EM group (Kruskal-Wallis; p =0.0264). (C) Seminal vesicle weight remained significantly lower in both EDS+Sham and EDS+EM groups whereas recovery is observed in the EDS+DIM group (1-way ANOVA; p = 0.0044). Shared letters above columns indicate no significant difference. n = 5-6 animals per group. (D) Representative multiplex genomic PCR for human and rat specific DNA (*HUMSAT17A*; 450bp and *Actb*; 552bp respectively) in testis biopsies 1 month after transplantation (EDS+35). No human DNA was detected in hAd-PSC transplanted testes. Genomic DNA isolated from EM and DIM cultured hAd-PSCs was included as a positive control.

5.2.2.3 Transplanted Cells do not engraft in the Rat Testis

Testicular histology was largely similar between groups at 35 days post EDS (Figure 5.6), with complete spermatogenesis taking place within seminiferous tubules, and abundant interstitial Leydig cells. However, in EDS+EM and EDS+DIM groups, occasional regions containing atrophic seminiferous tubules were observed. Given that this is not observed in the sham control groups, it is unlikely that these atrophic foci are a consequence of surgery or EDS toxicity.

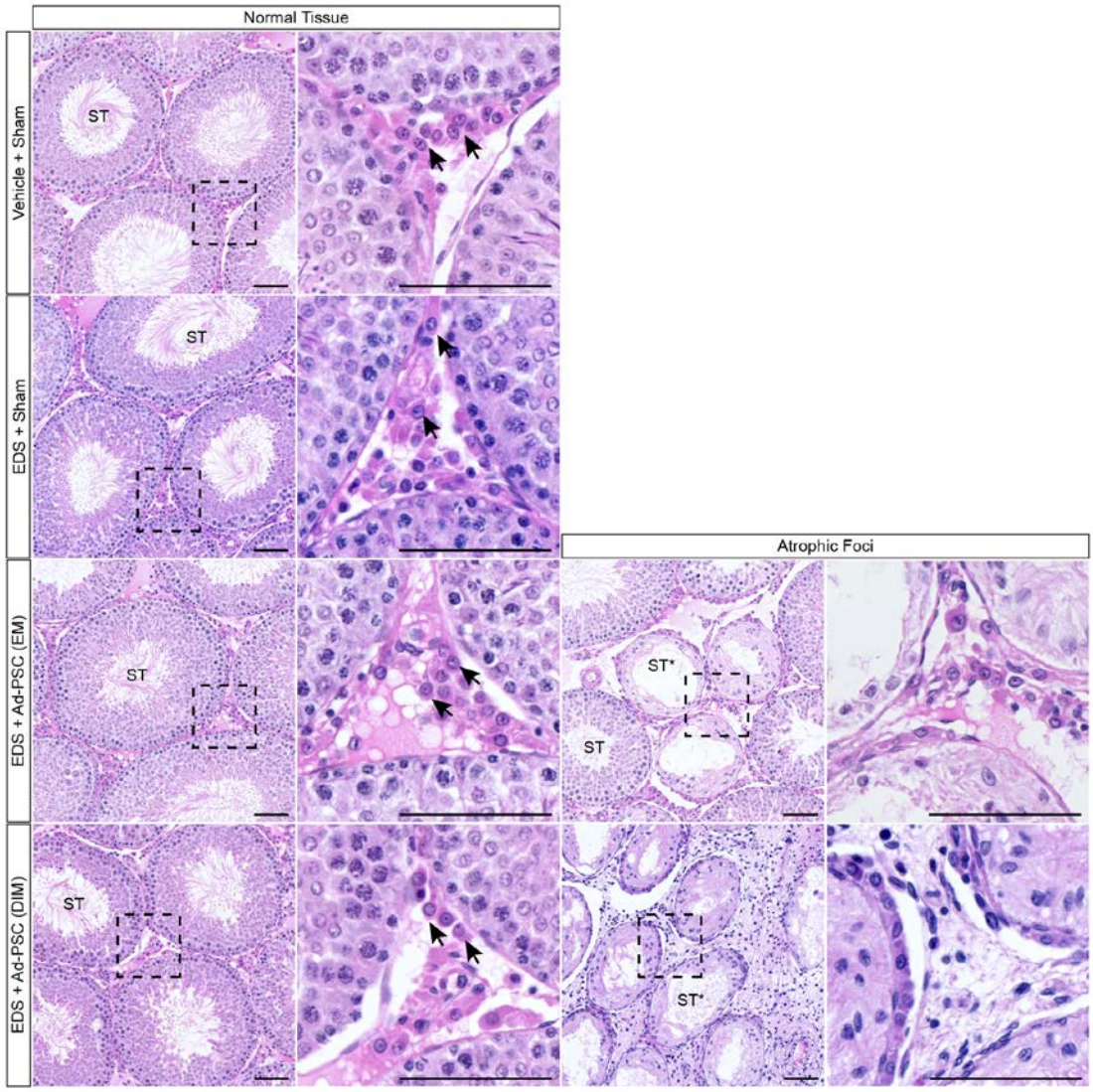


Figure 5.6 Testicular Histology following Leydig Cell Ablation and hAd-PSC Transplantation. Representative H&E stained testis sections revealed testicular architecture was largely normal 5 weeks post Leydig cell ablation. Full spermatogenesis within seminiferous tubules (ST) and abundant interstitial Leydig cells (arrows) were observed in all groups. However, regions containing atrophic tubules (ST*) and possible inflammatory cell infiltrates were noted in both EM and DIM hAd-PSC-transplanted testes. Scale = 100µm.

5.3 Discussion

The current strategy for treating hypogonadism is the administration of exogenous testosterone. However, this approach can have negative side effects, including disruption of normal spermatogenesis, which may not be desirable if maintenance of fertility is required (Samplaski *et al.*, 2014, McBride and Coward, 2016). Recent efforts have thus been focussed on developing stem cell-based regenerative therapies to restore endogenous androgen production as an alternative to exogenous testosterone

replacement (Peak *et al.*, 2016). In the experiments described herein, the regenerative capacity of human adipose-derived perivascular stem cells (hAd-PSC) to promote endogenous testosterone production in a rat model of Leydig cell ablation-regeneration was assessed. Following EDS-mediated Leydig cell ablation, hAd-PSCs were transplanted into the rat testis and Leydig cell regeneration was monitored *via* serial measurements of circulating luteinising hormone (LH) and testosterone (T). Alone, hAd-PSCs had negligible effects on Leydig cell regeneration kinetics. When cultured with luteinising hormone, insulin-like growth factor 1, platelet-derived growth factor-β and thyroid hormone the LH spike (induced by the removal of T negative feedback upon Leydig cell ablation) was dampened. This suggests the transplanted cells may promote the initial stages of Leydig cell regeneration. Notably, hAd-PSCs were undetectable in testis tissue five weeks after transplantation, suggesting they may not survive in the context of long-term xenogeneic transplantation but may initially provide paracrine support to endogenously regenerating Leydig cells.

In 2006, Ge *et al.* (2006) described a cocktail of hormones and growth factors for the *in vitro* generation of testosterone-producing Leydig-like cells from platelet derived growth factor alpha-positive stem cells isolated from the neonatal rat testis. More recently, Jiang *et al.* (2014) and Zang *et al.* (2017) used the differentiation inducing media to convert nestin-positive and CD51-positive testicular stem cells respectively into Leydig-like cells. Each study reported the induction of steroidogenic cell lineage markers including SF-1, GATA-4, LHCGR, STAR, CYP11A1, HSD3B6 and CYP17A1 after 7-11 days of culture in DIM. Whilst none demonstrated the expression of HSD17B3 – the enzyme required for the final step of testosterone biosynthesis – all reported the production of testosterone by cells cultured in DIM. In the current studies, only *STAR* and *CYP11A1* were detected in hAd-PSCs exposed to DIM strongly suggesting that they would not be able to produce testosterone with this differentiation regime. This may be explained by differences in the source of stem cells used. The above studies utilised stem cells isolated from the rodent testis. While both Jiang *et al.* (2014) and Zang *et al.* (2017) demonstrated multi-lineage potential of their respective stem cell populations, testicular stem cells may have an increased propensity to develop into Leydig cells. MSCs comprise a heterogeneous population of stromal cells, and heterogeneity of donor material as well as isolation and cultivation

procedures present another layer of complexity when characterising the regenerative potential of specific MSC subsets. Indeed, human stem cells isolated from different white adipose tissue depots, or even different sub-populations of stem cells from the same depot, are reported to have heterogenic differentiation potentials (Rada *et al.*, 2011, Lopa *et al.*, 2014, Russo *et al.*, 2014, Hindle *et al.*, 2017, Hardy *et al.*, 2017).

Leydig cell testosterone production is under negative feedback control by the hypothalamic-pituitary-gonadal (HPG) axis whereby pituitary-derived LH stimulates Leydig cell steroidogenesis, and increased circulating testosterone feeds back to the hypothalamic-pituitary unit to reduce LH secretion. Following Leydig cell ablation, this feedback loop is disrupted resulting in increased LH secretion (Morris *et al.*, 1986). Whilst transplantation of either EM or DIM cultured hAd-PSCs did not appear to impact recovery of circulating testosterone levels, the LH response in the EDS+DIM group was slightly decreased compared to that of the EDS+EM group. This may suggest that less LH may be required to drive testosterone synthesis by the regenerating Leydig cells when hAd-PSCs are 'primed' by exposure to DIM media prior to transplantation. Indeed, seminal vesicle weight - an accepted biomarker of peripheral androgen action (Welsh *et al.*, 2008, Mitchell *et al.*, 2015, van den Driesche *et al.*, 2015) was recovered in the EDS+DIM group by day 35 post EDS, indicative of increased peripheral androgen action compared to the EDS+Sham and EDS+EM groups.

Previous studies utilising stem Leydig cells isolated from rodent testes have reported successful engraftment and differentiation following EDS mediated Leydig cell ablation in immunocompetent hosts (Ge *et al.*, 2006, Jiang *et al.*, 2014, Zang *et al.*, 2017). However, these studies analysed testis sections only 10-12 days after transplantation, thus whether the cells survive long term engraftment remains to be established. Furthermore, the extent to which the transplanted cells contribute to the population of regenerated Leydig cells (both functionally and numerically) was not reported in any of these studies. In addition, none of the above studies measured circulating LH during the regeneration period thus the effect of the transplanted cells on the overall hypothalamic-pituitary-gonadal (HPG) axis activity is unknown. Zang *et al.* (2017) suggested that CD51-positive stem cells transplanted into the Leydig cell ablated rat testis produce testosterone under the regulation of the HPG-axis based on

pharmacological suppression of LH secretion by decapeptyl and subsequent reduction in circulating testosterone. However, the absence of a crucial control group treated with EDS and decapeptyl, but not transplanted with cells, severely limits the strength of this conclusion as the influence of decapeptyl on endogenously regenerating Leydig cells is not taken into account. Despite a modest effect of DIM cultured hAd-PSCs on circulating LH and seminal vesicle weight, hAd-PSCs were undetectable in the testis one month after transplantation, indicating that they do not engraft in the rat testis. As such, it is likely that any effect(s) may be due to paracrine support of endogenous regenerating Leydig cells, rather than their direct differentiation into Leydig cells. This is in line with previous reports of hAd-PSCs in a mouse model of ischaemic heart injury in which lineage tracing of GFP labelled hAd-PSCs transplanted into the myocardium demonstrated that only a very small number of cells differentiated into cardiac cells (Chen *et al.*, 2013). This suggests that the positive effects on cardiac recovery observed were, in this context, likely paracrine in nature. Similarly, adipose-derived stem cells were reported to have positive effects in a rat model of tobacco-induced erectile dysfunction by reducing oxidative stress (Huang *et al.*, 2016). EdU (5-ethynyl-2'-deoxyuridine) labelling of transplanted cells revealed minimal engraftment four weeks after injection leading the authors to speculate that paracrine release of cytokines/growth factors may be responsible for the therapeutic effect. Similarly, whilst Yang *et al.* (2015a) reported that adipose-derived stem cells could promote Leydig cell function in a pharmacologically induced ageing model, very few cells transplanted appeared to transform into Leydig cells. The authors suggested that the effect of the transplanted cells on testosterone production might be partly mediated by a reduction in reactive oxygen species.

Indeed, perivascular mesenchymal stem cells are reported to secrete a variety of factors including PDGF-BB (Chen *et al.*, 2009a) and LIF (Chen *et al.*, 2013), both of which have been suggested to play a role in stem Leydig cell proliferation/differentiation (Odeh *et al.*, 2014, O'Shaughnessy *et al.*, 2008). Furthermore, pre-incubation of primary rat Leydig cells with PDGF-BB has been shown to significantly enhance LH-stimulated testosterone synthesis (Risbridger, 1993) and, LIF has been suggested to influence testosterone production *in vitro* (Mauduit *et al.*, 2001, Wang *et al.*, 2016b). The precise extent to which hAd-PSCs support Leydig cell regeneration kinetics

requires further study. Nevertheless, these pilot studies represent the first description of the use of human adipose-derived perivascular stem cells in an *in vivo* model of Leydig cell injury, and suggest that these cells may exert a positive effect on Leydig cell regeneration by providing paracrine support.

6 General Discussion & Future Perspectives

In males, the testis is arguably the most important organ in the body - it produces germ cells through which genetic information is passed onto the next generation thus ensuring survival of the species. Additionally, it produces androgens which are required not only for sperm production, but also to support secondary sex characteristics and wider general health of the body such that adult males can function as suitable vehicles to carry the precious germ cell cargo. As males age, the testes atrophy and their spermatogenic and steroidogenic function becomes increasingly impaired. While maintenance of fertility may not be of primary concern to men approaching their twilight years, maintaining appropriate levels of circulating testosterone may be critically important to promote healthy ageing. It has been suggested that decreasing androgen levels in ageing men are linked to chronic age-related disorders including cardiovascular disease, metabolic syndrome, as well as decreased muscle mass and increased frailty (Kupelian *et al.*, 2006, Pye *et al.*, 2014, Yeap *et al.*, 2014, Antonio *et al.*, 2015). It has also been suggested that androgen levels are becoming increasingly lower in men compared with previous generations (Travison *et al.*, 2007, Andersson *et al.*, 2007, Perheentupa *et al.*, 2013). With an expanding ageing population in developed countries, it is essential the relationship between ageing, androgen levels and disease be further explored. Central to this is a well-developed understanding of the mechanisms that regulate proper testicular development and function, as well as the mechanisms by which testicular function (i.e. androgen production) deteriorates with age. As such, the main hypothesis behind the experiments carried out in the context of this thesis was that manipulation of the paracrine network in the testis *in vivo* would provide significant novel insights into the regulation of testicular function, uncovering potential therapeutic targets to treat age-related testicular dysfunction. To address this question, three sets of experiments were designed and carried out. Firstly, a series of novel mouse models of premature ageing were utilised to begin dissecting the process of age-related testicular degeneration, with the aim of establishing the cell specific contributions of premature ageing Leydig cells and Sertoli cells to the age-related decline in androgen production. Second, with the aim of determining the role(s) of leukemia inhibitory factor as a putative paracrine regulator of testis function, models of testicular cell-specific leukemia inhibitory factor

receptor ablation were generated and characterised. Finally, the impact of a stem cell-based regenerative therapy to promote endogenous testosterone production as a potential therapeutic strategy for the treatment of hypogonadism was determined.

6.1 Primary Hypogonadism in Prematurely Ageing *Cisd2*-deficient Mice

To date, the most widely used model for the study of age-related Leydig cell dysfunction has been the naturally aged brown Norway rat. This model has greatly increased our understanding of the changes occurring in aged Leydig cells including decreased LH-stimulated cAMP signalling and subsequent reduction in the expression and activity of enzymes involved testosterone biosynthesis. It has been suggested that these changes are partly mediated by cumulative cellular damage caused by reactive oxygen species (Beattie *et al.*, 2015). Other models of premature ageing which display primary hypogonadism including the D-galactose model (Ahangarpour *et al.*, 2014, Liao *et al.*, 2016) and the senescence accelerated SAMP8 mouse (Flood *et al.*, 1995, Smith *et al.*, 2013, Wang *et al.*, 2016a), both of which are associated with increased ROS production, provide support to this theory. However, one drawback with these models is that ageing occurs systemically, making it difficult to interpret cause and effect of specific testicular cell populations on Leydig cell dysfunction. To address this, the experiments detailed in Chapter 3 exploited the conditional potential of a novel *Cisd2* premature ageing allele to, for the first time, age Leydig cells and Sertoli cells separately. Intriguingly, whilst constitutive *Cisd2*-deficient mice displayed a phenotype reminiscent of age-related compensated Leydig cell failure characterised by a reduction in Leydig cell number, reduced expression of mRNAs encoding steroidogenic enzymes and a reduction in circulating testosterone (without decreased luteinising hormone); neither conditional *Cisd2*-deficient models recapitulated this phenotype. These novel data are in agreement with the suggestion of Chen *et al.* (2015) that age-related reductions in testosterone biosynthesis may be mediated by factors extrinsic to Leydig cells in aged animals and further suggest that the ageing Sertoli cell population may not directly contribute to age-related Leydig cell dysfunction.

Taking into account that conditional *Cisd2*-disruption, either in Leydig cells or in Sertoli cells alone, did not recapitulate the primary hypogonadism observed in constitutive CISD2-KO mice, the mechanisms underlying testicular degeneration and

Leydig cell dysfunction in this model of premature ageing are still unclear. At the cellular level, CISD2-loss causes endoplasmic reticulum and mitochondrial dysfunction and results in a pro-oxidative intracellular environment (Wiley *et al.*, 2013); processes which have been associated with impaired steroidogenesis (Allen *et al.*, 2006, Kim *et al.*, 2016, Huang *et al.*, 2017, Yang *et al.*, 2017a). Increased oxidative stress in the testes of constitutive *Cisd2*-KO mice, but not in either Leydig or Sertoli cell conditional *Cisd2*-KO, may explain the difference in phenotype between these models. Interestingly, Wiley *et al.* (2013) reported that the phenotype of *Cisd2*-deficient MEFs could be reversed by treatment with the antioxidant *N*-acetylcysteine. Additionally, it has been reported that antioxidant treatment can ameliorate Leydig cell damage under conditions of oxidative stress (Chen *et al.*, 2008, Ding *et al.*, 2017). As such, an interesting next step would be to assess (i) whether testicular redox status differs between the three premature ageing models and (ii) if an antioxidant treatment could ameliorate Leydig cell dysfunction in constitutive *Cisd2*-deficient mice as a potential therapeutic strategy to treat age-related primary hypogonadism.

A potential caveat to the prematurely aged Leydig cell model used herein is acknowledged. Whilst the lineage tracing studies using the *Pdgfrb*-Cre; tdTomato mice provide novel evidence that PDGFRb is a marker of adult Leydig stem/progenitor cells in fetal testis, this Cre targets only approximately 55 percent of adult Leydig cells. This observation adds weight to the suggestion that the adult testis harbours two distinct populations of Leydig cells (Payne *et al.*, 1980). However, it also raises the possibility that reduced testosterone production by *Cisd2*-deficient Leydig cells in the conditional model may be compensated for by the untargeted (wild-type or 'young') population. To address this, Leydig cell *Cisd2*-KO mice were treated with hCG to assess maximal testosterone production but no difference in Leydig cell response was noted. As such, this, together with the observation that LH is unaltered to maintain baseline testosterone, strengthens the conclusion that testosterone production is normal when premature ageing is restricted to Leydig cells.

Alterations in the wider endocrine milieu during ageing may result in diminished Leydig cell function. For example, increased circulating glucocorticoids have been reported in ageing rats (Sapolsky *et al.*, 1983, Lo *et al.*, 2000). It is well documented that glucocorticoids can modulate Leydig cell testosterone biosynthesis.

Administration of adrenocorticotrophic hormone (i.e. to stimulate adrenal glucocorticoid secretion) results in decreased circulating testosterone levels in men (Schaison *et al.*, 1978). Similarly, administration of exogenous hydrocortisone was shown to reduce circulating testosterone (Cumming *et al.*, 1983). Moreover, both these studies reported that the observed reduction in testosterone was not caused by altered LH secretion, suggesting a direct inhibitory effect of glucocorticoids on Leydig cells. Indeed, glucocorticoids have been shown to supress the expression and/or activity of LHCGR, StAR, CYP11A1, HSD3B and HSD17B in Leydig cells (Bambino and Hsueh, 1981, Badrinarayanan *et al.*, 2006, Martin and Tremblay, 2008). Very preliminary data (thus not reported herein) suggest that circulating corticosterone may be elevated in the constitutive *Cisd2*-KO mice, but not in either conditional model. It is therefore tempting to speculate that increased Leydig cell glucocorticoid signalling may underlie the reduced testosterone production in constitutive *Cisd2*-KO mice. Further experiments are required to test this hypothesis.

Degeneration of the seminiferous tubules is a common feature observed in the ageing testis. Previous reports suggest that Sertoli cell number and/or function declines with advancing age (Neaves *et al.*, 1984, Tenover *et al.*, 1988, Johnson *et al.*, 1984a, Johnson *et al.*, 1990, Mahmoud *et al.*, 2000). Consistent with these reports, constitutive *Cisd2*-deficient mice displayed signs of testicular atrophy accompanied by reductions in seminiferous tubule diameter, Sertoli cell number and decreased numbers of sperm in the cauda epididymis. However, circulating FSH was not measured in these animals, which would provide additional insight into Sertoli cell function in this model of premature ageing. Sertoli cells play a critical role in supporting spermatogenesis and in regulating the activity of peritubular myoid cells that contribute to the architecture and function of the seminiferous tubules (Rebourcet *et al.*, 2014b). As such, it was anticipated that prematurely aged Sertoli cells in the conditional model would result in a similar degeneration of the seminiferous epithelium. However, this was not the case thus questioning a direct role of ageing Sertoli cells on tubular degeneration. It is possible that the aged germ cells/and or peritubular cells mediate aspects of age-related seminiferous tubule degeneration in constitutive *Cisd2*-deficient mice. Additionally, Paniagua *et al.* (1991) suggested that age-associated involution of the seminiferous tubules may be related to altered testicular vascular function. As such, investigations

into peritubular myoid cell activity/function as well as the testicular microvasculature network in prematurely ageing *Cisd2*-deficient mice may be interesting avenues for future exploration.

6.2 Sertoli Cells are a Major Target of Leukemia Inhibitory Factor Signalling in the Testis

A complex paracrine signalling network supports overall testis function by facilitating crosstalk between various cellular components to orchestrate the processes of spermatogenesis and steroidogenesis. A detailed understanding of how testis homeostasis is regulated at the paracrine level could inform on how testis function fails in cases of reduced fertility, disease and during the ageing process. To this end, the experiments detailed in chapter 4 investigated the role of leukemia inhibitory factor signalling in the testis. Several reports have detailed the expression of LIF and LIFR/gp130 signalling subunits in the testis, both in fetal life and in adulthood (Jenab and Morris, 1998, Piquet-Pellorce *et al.*, 2000, Molyneaux *et al.*, 2003, Dorval-Coiffec *et al.*, 2005). Interestingly, Piquet-Pellorce *et al.* (2000) reported that peritubular myoid cells are the principal source of LIF within the testis and proposed that LIF is a putative paracrine regulator of normal testicular development and function. However, to date, a definitive role for LIFR signalling in the testis *in vivo* has not been described.

To address the question of how LIF/LIFR signalling contributes to testis function, novel models of conditional LIFR ablation were generated. Firstly, because LIFR has been detected in germ cells from the spermatogonial stage and LIF has been suggested to promote spermatogonial proliferation or survival *ex vivo/in vitro* (De Miguel *et al.*, 1996, Dorval-Coiffec *et al.*, 2005), the effect of conditional germ cell LIFR ablation was investigated. Surprisingly, spermatogenesis appeared to proceed normally in germ cell LIFR-KO mice demonstrating that germ cell autonomous LIFR signalling is dispensable for fertility. As such, the question of how the previously suggested influence of LIF on spermatogonial proliferation/survival may be mediated remained open. Both *in vitro* systems utilised by De Miguel *et al.* (1996) and Dorval-Coiffec *et al.* (2005) contained Sertoli cells. This raised the possibility that the observed effects of LIF on spermatogonia are mediated *via* LIFR signalling in Sertoli cells as had previously been hypothesised by Jenab and Morris (1998). Indeed, conditional LIFR

ablation from the Sertoli cell population resulted in a reduction in spermatogonia and Sertoli cells and was accompanied by a progressive, degenerative phenotype characterised by abnormal germ cell loss, sperm stasis, seminiferous tubule distention and atrophy of the seminiferous tubules. Taken together, the results from both conditional LIFR ablation models provide novel *in vivo* evidence to support the hypothesis that the effect of LIF on germ cells are mediated *via* Sertoli cells.

Further experiments are required to establish the mechanisms by which Sertoli cell LIFR signalling supports spermatogenesis. It has been demonstrated *in vitro* that the Jak/STAT pathway may function downstream of LIFR in Sertoli cells *via* phosphorylation of STAT3 (Jenab and Morris, 1998). Therefore, it would be interesting to assess whether disruption to this pathway underlies the phenotype observed in the Sertoli cell LIFR-KO testis. For example, a conditional STAT3 allele could be utilised to disrupt Sertoli cell STAT3 and the phenotype compared to that of the Sertoli cell LIFR-KO. Conversely, overexpression of STAT3 in the Sertoli cell LIFR-KO testis (i.e. using a Sertoli cell targeting lentivirus) may rescue the phenotype.

Leukemia inhibitory factor receptor is also expressed in stem and adult Leydig cells (Jenab and Morris, 1998, Dorval-Coiffec *et al.*, 2005, Ge *et al.*, 2006, Jiang *et al.*, 2014). It has been reported previously that LIF can stimulate the proliferation of stem Leydig cells isolated from the neonatal rat testis (Ge *et al.*, 2006) and *Lif* mRNA expression is transiently upregulated in the initial stages of Leydig cell differentiation following EDS-mediated Leydig cell ablation (O'Shaughnessy *et al.*, 2008), together suggesting a role for LIFR in Leydig cell development. Additionally, *in vitro* studies have demonstrated LIF can have both inhibitory and stimulatory effects on Leydig cell and adrenal steroidogenesis (Mauduit *et al.*, 2001, Bamberger *et al.*, 2000, Mikhaylova *et al.*, 2008, Wang *et al.*, 2016b). However, a definitive role for LIFR signalling in Leydig cell development and function *in vivo* has not yet been described. An attempt was made during the course of this PhD project to ablate LIFR from the Leydig cell population using the *Ap2*-Cre which targets adult Leydig cells from the stem/progenitor stage (Kilcoyne *et al.*, 2014, O'Hara *et al.*, 2015). Unfortunately, this experiment was abandoned as it became apparent that disruption of *Lifr* using this Cre could possibly result in lethality due to off target effects in the nervous system (Martens *et al.*, 2010). Recently, Penny *et al.* (2017) reported that adenoviral vectors

could transduce Leydig cells *in vivo* when injected into the mouse testis interstitium and, when packaged with Cre, could induce recombination of conditional alleles. As such, experiments are now underway in the lab to determine whether this approach could be used to disrupt Leydig cell *Lifr* expression in the testes of mice carrying the conditional *Lifr* allele.

6.3 Regenerative Potential of Human Adipose-derived Perivascular Stem Cells to Support Leydig Cell Function

The identity and behaviour of stem Leydig cells has been an area of intense research over recent years, particularly in relation to harnessing their regenerative properties as a potential treatment for androgen deficiency. The precise origin of stem Leydig cells within the testis is debated, with both peritubular and perivascular origins proposed. In relation to the latter, Davidoff *et al.* (2004) suggested that Leydig cells arise from perivascular pericytes (characterised by NG2 and PDGFRb expression) following EDS-mediated Leydig cell ablation. In attempt to clarify the identity of mesenchymal stem cells present in multiple tissues, Crisan *et al.* (2008) proposed a universal identity of perivascular stem cells in a wide panel of human organs. Specifically, the authors suggest that multipotent perivascular stem cells are identifiable by their expression of NG2, PDGFRb and CD146 as well as a number of mesenchymal stem cell markers including CD90. Interestingly, expression of each these markers has been documented in putative stem Leydig cells in the rodent testis (Davidoff *et al.*, 2004, Jiang *et al.*, 2014, Li *et al.*, 2016). Additionally, the Leydig cell lineage tracing carried out in this thesis (chapter 3) suggest that a sub-population of adult Leydig cells arise from PDGFRb expressing cells. As such, the experiments carried out in chapter 5 assessed whether perivascular stem cells isolated from human adipose tissue (hAd-PSCs) had the potential to promote Leydig cell regeneration following EDS-mediated ablation.

A cocktail of growth factors and hormones have previously been reported to induce the differentiation of stem Leydig cells *in vitro*. This was first described by Ge *et al.* (2006) who suggested that a combination of LH, IGF-1, PDGF-B and T_3 (which have been suggested to influence adult Leydig cell development) was sufficient to convert rat testicular stem cells into steroidogenic Leydig cells. This differentiation inducing medium (DIM) was subsequently reported to transform multipotent stem cells isolated

from the mouse testis into Leydig cells (Jiang *et al.*, 2014, Zang *et al.*, 2017). Whether this protocol could be used to differentiate multipotent human stem cells of an extra gonadal origin was addressed herein. Culturing hAD-PSCs in DIM was not sufficient to promote their differentiation into Leydig cells *in vitro*. However, *STAR* and *CYP11A1* were upregulated, which may indicate commitment of these cells to a steroidogenic lineage. Further experiments are required to explore this possibility. For example, adding additional factors to the differentiation inducing media may promote the transformation of hAd-PSCs into Leydig cells. Both PDGF-A and DHH would be interesting candidates to test as both have been suggested to play a role in adult Leydig cell development (Clark *et al.*, 2000, Gnessi *et al.*, 2000, O'Shaughnessy *et al.*, 2008). Importantly, Odeh *et al.* (2014) reported that whilst both PDGF-A and PDGF-B could stimulate stem Leydig cell proliferation, PDGF-A stimulated differentiation whereas PDGF-B was inhibitory.

To further explore whether hAd-PSCs could form Leydig cells, they were transplanted into the rat testis following EDS-mediated Leydig cell ablation (i.e. in an environment conducive to stem Leydig cell differentiation). However, when hAd-PSC transplanted testes were analysed after 5 weeks, there was no evidence of engraftment. Interestingly, after 5 weeks, seminal vesicle weight (as a marker of peripheral androgen action) was similar between Vehicle+Sham control animals (i.e. no Leydig cell ablation and no cell transplant) and animals that received hAd-PSCs cultured in DIM. Additionally, DIM-exposed hAd-PSCs had a modest effect on circulating LH levels during the initial 2 weeks post EDS. This suggested that DIM-exposed hAd-PSCs might promote Leydig cell regeneration. It would be interesting to repeat this experiment, harvesting testes at 7, 14 and 21 days post EDS to determine the mechanism by which DIM cultured hAd-PSCs may alter regeneration kinetics. For example, it may be the case that these cells differentiate into functional Leydig cells prior to being lost from the testis, or that they enhance the proliferation and/or differentiation of endogenously regenerating Leydig cells.

6.4 Conclusions

The main hypothesis behind the experiments carried out in the context of this thesis was that manipulation of the paracrine network in the testis *in vivo* would provide significant novel insights into the regulation of testicular development and function, uncovering potential therapeutic targets to treat age-related testicular dysfunction. Several novel observations herein support this hypothesis:

1. Intrinsic ageing in Leydig and Sertoli cells *per se* may not directly contribute to age-related testicular atrophy and Leydig cell dysfunction. As such, it is suggested that disruption to the testicular microenvironment and/or wider systemic effects of ageing play a significant role in age-related testicular dysfunction.
2. Leukemia inhibitory factor receptor signalling is required in Sertoli cells but not in germ cells to support normal function of the seminiferous tubules (i.e. to maintain spermatogenesis).

Additional novel observations reported herein include:

1. Constitutive *Cisd2*-deficient mice represent a novel model of age-related primary hypogonadism with utility for the expedited study of age-related testicular dysfunction.
2. A sub population of adult Leydig cells arise from PDGFRB positive stem/progenitor cells present in the fetal testis.
3. Leukemia inhibitory factor receptor is dispensable for normal testicular development during fetal life.
4. Human adipose-derived stem cells may promote Leydig cell regeneration following EDS-mediated ablation.

In summary, the studies reported herein further refine our understanding of the complex crosstalk between individual cell types within the testis, which may have wider implications for the development of novel therapeutics to treat testicular dysfunction in cases of infertility and during the ageing process.

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